REMARKS

By way of amendment, claims 38 and 40-87 are cancelled. New claims 81-117 are hereby added. Claim 81 is a product-by-process claim that finds support in the specification at, *inter alia*, page 37-39 (Example 1), and on page 4, line 34 to page 5, line 15. Claims 82-117 find support throughout the specification, particularly at pages 28-33. No new matter has been added.

Sequence Listing

The Examiner points out that the instant application is not in compliance with the sequence rules under 37 C.F.R. §§ 1.821-1.825. Applicants have corrected the errors indicated in the error report and herewith include a corrected sequence listing in both printed and computer readable formats.

Priority

The Examiner's prior art rejections under 35. U.S.C. §102 and §103 presume an effective filing date subsequent to that of Ni *et al.* (Publication No. 20020098550). Applicants respectfully disagree.

The Examiner contends that the "02/13/1997 disclosure (ASN: 08/799,861) fails to provide adequate written description because a skilled artisan would not know how to make the claimed antibody based on the information provided." The Examiner alleges that the skilled artisan would attempt to raise antibodies using the peptide sequences VPANEGD, VCEV, or SGEVELSSV and that since the latter two are not found in SEQ ID NO: 2 their use would hinder the process. The Examiner also argues that the sequence VPANEGD closely resembles the sequence VPANGAD that is part of DR4 (i.e., TRAIL-R1 receptor) and that reliance on the peptide VPANEGD alone to raise an antibody specific for the polypeptide of SEQ ID NO: 2 would require undue experimentation.

As for Applicants' filing of March 12, 1997, the Examiner states that it is not clear that the 51-mer of SEQ ID NO: 4 and the sequence VPANEGD are actually part of the same polypeptide chain. The Examiner concludes that it appears that "Applicants were in possession of a mixture of partially characterized polypeptides that may have included the polypeptide encoded by SEQ ID NO: 2 and that the disclosure dated 03/12/1997 neither provides a written description for the polypeptide encoded by SEQ ID NO: 2 nor enables one of skill in the art to make and identify the antibodies claimed in the instant application". Before specifically addressing the Examiner's comments, Applicants wish to correct some misimpressions with respect to TRAIL-R and TRAIL binding proteins in general.

1. Five TRAIL Receptor Proteins - Only OPG is Not a Cell Surface Receptor

   TRAIL is a ligand that binds to five distinct TRAIL receptors. These receptors are: TRAIL-R1 (also known as DR4), TRAIL-R2 (DR5 or "TRAIL-R" in the instant application), TRAIL-R3 (DcR1), TRAIL-R4 (DcR2), and osteoprotegerin (OPG). All of these TRAIL receptors, with the exception of OPG, are cell surface receptors. OPG is a soluble TRAIL binding protein. See, for example, Shipman & Croucher, *Cancer Research*, 63: 912-016 (2003) "Osteoprotegerin is a Soluble Decoy Receptor for

Tumor Necrosis Factor-related Apoptosis-inducing Ligand/Apo2 Ligand and Can Function as a Paracrine Survival Factor for Human Myeloma Cells".

2. OPG is Not Isolated from Jurkat Cell Plasma Membranes

Claim 81 is a product-by process claim to TRAIL-R (TRAIL-R2) receptor protein isolated and purified from plasma membranes of Jurkat cells. The isolation and purification method is disclosed in Example 1 of the instant application as well as of that filed on February 13, 1997. OPG is a soluble protein and would not be a component of plasma membranes. Consequently, it would not be present in the product made by the process of claim 81.

3. TRAIL-R1 (DR4) is Not Expressed on the Surface of Jurkat Cells

Applicants' representative has identified eleven references (cited below and enclosed herewith as Exhibits A, B, and D-L) that experimentally assess TRAIL-R1 protein expression by Jurkat cells. Nine of these eleven references (Exhibits D-L) demonstrate that Jurkat cells do not express TRAIL-R1 protein. The two contrary references (Exhibits A & B) fail to control for cross-reactivity to TRAIL-R2 (TRAIL-R).

False positives stemming from cross-reactivity of anti-TRAIL-R1 antibodies to TRAIL-R2 protein is a source of error recognized in the literature. For example, Exhibit C (enclosed) teaches TRAIL-R1 antisera and notes, "[i]n western blots, this antibody shows approximately 10% cross-reactivity with rhTRAIL R2 . . ." (emphasis added). Muhlenbeck *et al.* (Exhibit L) notes problems of "significant cross-reactivity" between anti-TRAIL-R1 antibodies and TRAIL-R2 protein. At page 32209, left column, second paragraph. These references demonstrate that the conclusions drawn by Exhibits A and B are unreliable; they lack proper controls to address the problem of false positives.

In contrast, Applicants draw the Examiner's attention to the evidence provided by nine references (Exhibits D-L) that demonstrate that Jurkat cells do not express TRAIL-R1 protein. Applicants also note the superior experimental designs of such references as Sprick *et al.*, Matysiak *et al.*, or Muhlenbeck *et al.* each of which anticipates potential problems from false positives by controlling for cross-reactivity or otherwise ensuring antibody specificity to TRAIL-R1. From the results obtained by these nine separate studies, taken together with the deficiencies in the experimental designs of Exhibits A and B, one can conclude with a high degree of confidence that TRAIL-R1 protein is not expressed on Jurkat cell membranes. In short, Applicants' disclosure of February 13, 1997 does not (and could not) enable an admixture of TRAIL-R2 and TRAIL-R1.

Exhibit D
Title: FADD/MORT1 and Caspase-8 Are Recruited to TRAIL Receptors 1 and 2 and Are Essential for Apoptosis Mediated by TRAIL Receptor 2
Authors: Sprick *et al.*
Journal: Immunity, 12: 599-609 (2000)

Results: "While BL60, BJAB, and CEM cells expressed both apoptosis-inducing TRAIL receptors, only TRAIL-R2 was present on the surface of Jurkat cells (Figure 1C, top panels). TRAIL-R3 and TRAIL-R4 was not detectable on the surface of these cells lines (Figure 1C, bottom panels)." Page 600, right column, lines 28-33.

Exhibit E
Title: TRAIL induces death of human oligodendrocytes isolated from adult brain
Authors: Matysiak *et al.*
Journal: Brain, 125: 2469-2480 (2002)
Results: "We used western blotting and flow cytometry . . . the Jurkat cell line expressed only TRAIL-R2 and a low level of TRAIL-R3 both on the cell surface and intracellularly." Page 2475, left column, second paragraph.

Exhibit F
Title: Antileukemic drugs increase death receptor 5 levels and enhance Apo-2L-induced apoptosis of human acute leukemia cells
Authors: Wen *et al.*
Journal: Blood, 96 (12): 3900-3906 (2000)
Results: "Jurkat cells, which demonstrated the highest sensitivity to Apo-2L, expressed higher levels of DR5, but lacked the expression of DR4 (Figure 1C)." Page 3902, right column, second paragraph.

Exhibit G
Title: TRAIL/Apo-2 Ligand Induces Primary Plasma Cell Apoptosis
Authors: Ursini-Siegel *et al.*
Journal: J. Immunology, 169:5505-5513 (2002)
Results: "The maintenance of DR4 and DR5 expression was confirmed at the protein level by immunoblot analysis (Fig. 2B), where detection of DR5 but not DR4 in Jurkat T cells served as a control for the Ab specificity." Page 5507, right column.

Exhibit H
Title: TRAIL receptor-2 signals apoptosis through FADD and caspase-8
Authors: Bodmer *et al.*
Journal: Nature Cell Biology, 2: 241-243 (2000)
Results: ". . . we next analysed Jurkat T cells, which have been shown previously to express TRAIL-R2 but not TRAIL-R1, cDNA. In agreement with this, we found that only TRAIL-R2 bound to immunoprecipitated TRAIL in Jurkat cells (Fig. 1b)." Page 242, left column, second full paragraph.

Exhibit I
Title: Molecular requirements for the combined effects of TRAIL and ionizing radiation
Authors: Jendrossek *et al.*

Journal: Radiother. Oncol. 68(2):189-198 (2003)
"Surface expression of TRAIL receptors DR4 and DR5 was analysed by FACS. Results: "Jurkat T-cells express the agonistic DR5 receptor but not DR4."

Exhibit J
Title: Mitogen-Activated Protein Kinase/Extracellular Signal-Regulated Kinase Signaling in Activated T Cells Abrogates TRAIL-Induced Apoptosis Upstream of the Mitochondrial Amplification Loop and Caspase-8
Authors: Soderstrom *et al.*
Journal: J. Immunol., 169: 2851-2860 (2002)
Results: " . . . we analyzed the relative amount of DR4 and DR5 on the surface of Jurkat T cells. Jurkat T cells were immunofluorescence labeled with mAbs to the two respective receptors and analyzed by flow cytometry. The results show that predominantly DR5 is expressed on Jurkat T cells . . .". Page 2854, right column, second paragraph. See, Figure 4 showing no difference between control and DR4 on Jurkat T cells. Note: Alexis Biochemicals antibody used in this study (See, Exhibit K)

Exhibit K
Title: Product Data Sheet from Alexis Biochemicals "Monoclonal Antibody to TRAIL-R1 (human)" ALX-804-297
Results: "Flow cytometric detection of endogenous TRAIL-R1 surface expression on BJAB cells versus a TRAIL-R1 negative cell line, Jurkat."

Exhibit L
Title: The Tumor Necrosis Factor-related Apoptosis-inducing Ligand Receptors TRAIL-R1 and TRAIL-R2 Have Distinct Cross-linking Requirements for Initiation of Apoptosis and are Non-redundant in JNK Activation
Authors: Muhlenbeck *et al.*
Journal: Journal of Biological Chemistry, 275 (41): 32208-32213 (2000)
Results: "As shown in Fig. 4A, all cells investigated with the exception of Jurkat cells were positive for TRAIL-R1 . . ." Page 32212, left column, second paragraph. Note: See discussion of cross-reactivity between anti-TRAIL-R1 and anti-TRAIL-R2 antibodies at page 32209, left column, second paragraph.

4. TRAIL-R3 (DcR1) is Not a Component of the Claimed Protein

Assuming, *arguendo*, that TRAIL-R3 (DcR1) is present on Jurkat cell membranes, Applicants note that the molecular weight of TRAIL-R3 (Exhibit M) is in the range of 32-35 kDa. In contrast, the claimed invention recites a size limitation of "about 50 to 55 kilodaltons" (corresponding to a size of over 400 amino acids for TRAIL-R). This size range cannot reasonably be argued to embrace the size of TRAIL-R3 (32-35 kDa, 259 amino acids in length). Additionally, the claimed invention is limited to the isolated protein comprising the amino acid sequence VPANEGD, a sequence that lacks any match in TRAIL-R3. With the exception of TRAIL-R2, none of the TRAIL binding proteins has even one of these recited structural elements let alone both. Given the

substantially distinct structural features of TRAIL-R2 and TRAIL-R3, it is clear that Applicants' disclosure of February 13, 1997 enables one of ordinary skill in the art to isolate and purify the claimed TRAIL-R2 apart from TRAIL-R3 (e.g., by SDS-PAGE). The molecular weight and peptide sequence that Applicants obtained from the isolated and purified protein from Jurkat cells is itself supportive of Applicants claim that they were in possession of TRAIL-R2 alone.

5. TRAIL-R4 (DcR2) is Not a Component of the Claimed Protein

Applicants are unaware of any evidence that TRAIL-R4 is expressed on the cell surface of Jurkat cells. On the contrary, Matysiak *et al.* (Exhibit L) investigated cell surface expression from a number of cell types, including Jurkat cells. It states that the "expression of TRAIL-R4 on the cell surface was absent from all the studied cell populations . . ." At p. 2475. Applicants also note that the molecular weight of TRAIL-R4 is approximately 35 kDa (Exhibit N), substantially different from that of TRAIL-R2. TRAIL-R4 receptor also lacks any match to the TRAIL-R2 sequence VPANEGD. Consequently, given the substantial structural differences (e.g., size and sequence) between TRAIL-R2 and TRAIL-R4, it is clear that Applicants' disclosure of February 13, 1997 readily enables one of ordinary skill in the art to isolate and purify TRAIL-R2 apart from TRAIL-R4 even if TRAIL-R4 were present on Jurkat cell membranes.

6. The Claimed TRAIL-R (TRAIL-R2) is Enabled by the Filing of February 13, 1997

The Federal Circuit has held that the correct standard of proof needed to establish priority is the "clear and convincing" standard. Price v. Symsek, 988 F.2d 1187, 26 USPQ2d 1031 (Fed. Cir. 1993). "Clear and convincing" evidence has been described as evidence which produces in the mind of the trier of fact an abiding conviction that the truth of a factual contention is "highly probable." Colorado v. New Mexico, 467 U.S. 310, 316, 104 S.Ct. 2433, 2437, 81 L.Ed.2d 247 (1983).

Applicants submit that one can conclude with requisite high probability that the claimed isolated and purified TRAIL-R2 (TRAIL-R) was enabled by Applicants' disclosure of February 13, 1997. Applicants have provided evidence that of the five TRAIL binding proteins only TRAIL-R3 could be present with TRAIL-R2 on the surface of Jurkat cell membranes. And, given the substantial structural differences between TRAIL-R2 and TRAIL-R3, one of ordinary skill in the art could readily use the teachings of Applicants' disclosure of February 13, 1997 to isolate and purify the claimed TRAIL-R2 protein apart from TRAIL-R3 (e.g., by size separation using SDS-PAGE). Indeed, the molecular weight and sequence obtained from the protein isolated per the method of Example 1 (and as claimed in claim 81) is fully consistent with Applicants' position that they were in possession of TRAIL-R2, not a partially characterized mixture further comprising TRAIL-R3 (or any other TRAIL binding protein). In short, the evidence overwhelmingly favors an inference that Applicants both enabled and were in possession of the isolated and purified TRAIL-R2 receptor protein of claim 81. As a corollary, since Applicants were in possession of the claimed TRAIL-R protein, as of February 13, 1997 they also enabled and provided written description for the claimed plurality of antibodies to TRAIL-R (TRAIL-R2). See, Noelle v. Lederman, 355 F.3d 1343 (Fed. Cir. 2004). Clearly, with TRAIL-R2 isolated and purified, one of ordinary skill in the art would not have used the peptide sequences VCEV or SGEVELSSV (obtained from PS-1 cells) to

generate the claimed antibodies when isolated and purified TRAIL-R2 from Jurkat cells is enabled, claimed, and, constitutes a recited element of the claimed antibodies. Applicants request that the Examiner rightfully assign an effective priority date of February 13, 1997 to the claimed invention.

35 U.S.C. §102

Claims 38, 40-52, and 60-73 were rejected under 35 U.S.C. §102(e) as being anticipated by Ni *et al.*, Publication No. 20020098550 (Application No. 10/005842). The Examiner contends that Ni *et al.* discloses an alternative splice variant (411 amino acids) of DR5 (TRAIL-R; TRAIL-R2). The Examiner states that the polypeptide and nucleic acid sequences of DR5 as well as antibodies to DR5 are fully disclosed in priority document, provisional patent application 60/040846, filed March 17, 1997. Applicants respectfully traverse.

For reasons discussed in detail above, the effective filing date for the claimed invention is February 13, 1997. The application of Ni *et al.* is thus antedated by Applicants' effective filing date and Ni *et al.* cannot properly be cited as prior art against the pending claims. The suggestion that Applicants merely had in their possession a partially characterized mixture of TRAIL binding proteins is inconsistent with the substantial weight of theoretical and empirical evidence. On the contrary, from an objective appraisal of the totality of the evidence one can conclude with a high degree of confidence that isolated and purified TRAIL-R (TRAIL-R2) was enabled by Applicants' disclosure of February 13, 1997 and, likewise, was in their possession.

Applicants note that any prior art rejection alleging that a reference disclosing an antibody to TRAIL-R1 inherently discloses an antibody that would specifically bind to TRAIL-R2 (due to cross-reactivity) is obviated by the recitation in the current claims of a plurality of anti-TRAIL-R antibodies wherein each of the antibodies in the plurality specifically binds to (or is bound to) TRAIL-R. None of the cited references (or any reference disclosing anti-TRAIL R1 antibodies) teach or suggest a plurality of anti-TRAIL-R2 antibodies with the recited functional characteristics. Accordingly, Applicants believe that the rejection made under §102(e) is without merit. Applicants courteously request its withdrawal.

35 U.S.C. §103

Claims 53-59 were rejected under 35 U.S.C. §103(a) as being unpatentable over Ni *et al.* (discussed previously), in further view of Hoogenboom *et al.* Patent No. 5,565,332. Hoogenboom *et al.* is cited as teaching humanization of monoclonal antibodies. The Examiner states that it would have been obvious to one of skill in the art at the time the instant invention was made to combine the teachings of Ni *et al.*, which provide antibodies to the DR5/TRAIL-R polypeptide with the teachings of Hoogenboom *et al.*, to provide humanized antibodies, with a reasonable expectation of success. Applicants respectfully traverse.

As indicated previously, Applicants effective filing date for the claimed invention is properly February 13, 1997, thus antedating the filing date of Ni *et al.* Ni *et al.* is not properly citable as prior art. Accordingly, Applicants respectfully request withdrawal of this rejection.

Conclusion

Applicants believe that the formal matters and grounds for which the claimed invention was rejected have now been addressed and respectfully request that a timely Notice of Allowance be issued for this case. The Examiner is invited to call the undersigned to resolve any outstanding issues.

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Law Department
1201 Amgen Court West
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Telephone: (206) 587-0430

Respectfully submitted,

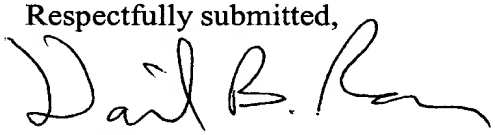

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EXHIBIT A

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ΨProSci INCORPORATED

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Anti-DR4 (NT) *TRAIL-R1*

CATALOG No.: 1167

BACKGROUND:

Apoptosis, or programmed cell death, occurs during normal cellular differentiation and development of multicellular organisms. Apoptosis is induced by certain cytokines including TNF and Fas ligand in the TNF family through their death domain containing receptors, TNFR1 and Fas. A novel death domain containing receptor was recently identified and designated DR4 (for death receptor 4)[1]. The ligand for this novel death receptor has been identified and termed TRAIL[2,3], which is a new member in the TNF family. DR4 is also called TRAIL receptor-1 (TRAIL-R1)[4]. DR4 is expressed in most of human tissues including spleen, peripheral blood leukocytes, small intestine and thymus. Like TNFR1, Fas and DR3, DR4 mediates apoptosis and NF-κB activation in many tissues and cells.

SOURCE:

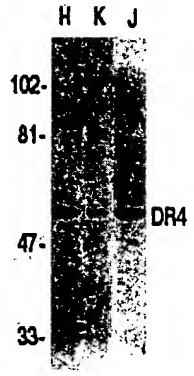
Rabbit anti-DR4 (NT) polyclonal antibody was raised against a peptide corresponding to amino acid 1 to 20 of human DR4 mature protein.

APPLICATION:

This polyclonal antibody can be used for detection of DR4 by Western blot at 1:500 to 1:1000 dilution. HeLa or Jurkat whole cell lysate can be used as positive control and a 57 kDa band can be detected. For research use only.

STORAGE:

Supplied as purified IgG, 100 µg in 200 µl of PBS containing 0.02% sodium azide. Store at 4°C, stable for one year.



Western blot analysis of DR4 in HeLa (H), K562 (K), and Jurkat (J) whole cell lysate with anti-DR4 (NT) at 1:500 dilution.

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(RD1299)

EXHIBIT B

Pancreas
Vol. 23, No. 1, pp. 72–79

Pancreatic Adenocarcinoma Cell Lines Show Variable Susceptibility to TRAIL-Mediated Cell Death

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Background and Aims: Programmed cell death via the Fas receptor/Fas Ligand and DR4, DR5/TRAIL plays a major role in tumor escape and elimination mechanisms. It also promises to be an effective therapy alternative for aggressive tumors, as has been recently shown for colon, breast, and lung cancer cells. We attempted to clarify the role of these molecules in aggressivity of pancreatic carcinomas and to identify possible pathways as targets for therapy.
Methods: Five pancreatic cell lines were investigated for the expression of FasL/Fas, DcR3, DR4, DR5/TRAIL, DcR1, DcR2, and other death pathways related molecules such as Bax, bcl-xL, bcl-2, FADD, and caspase-3 by flow cytometry, immunoblotting, and RT/PCR, both semiquantitative and real time (TaqMan). The susceptibility of these cell lines to apoptosis mediated by recombinant TRAIL was investigated. The effect of therapeutic agents (gemcitabine) on their susceptibility to TRAIL induced apoptosis was studied as well.
Results: Pancreatic adenocarcinomas expressed high levels of apoptosis-inducing receptors and ligands. They showed differential susceptibility to cell death induced by TRAIL, despite expressing intact receptors and signaling machineries. Treatment with commonly used therapeutic agents did not augment their susceptibility to apoptosis. This could be explained by the fact that they expressed differentially high levels of decoy receptors, as well as molecules known as inhibitors of apoptosis.
Conclusions: The data suggest that pancreatic carcinoma cells have developed different mechanisms to evade the immune system. One is the expression of nonfunctional receptors, decoy receptors, and molecules that block cell death, such as bcl2 and bcl-xL. The second is the expression of apoptosis-inducing ligands, such as TRAIL, that could induce cell death of immune cells. The success in treating malignant tumors by recombinant TRAIL might apply to some but not all pancreatic tumors because of their differential resistance to TRAIL-induced cell death. **Key Words:** Pancreatic carcinoma—Apoptosis—TRAIL—Chemotherapy—FasL.

Pancreatic carcinomas are highly aggressive tumors with a poor prognosis (1). They rank fourth among cancer-related deaths (2,3), partly because they do not respond substantially to radiation and/or chemotherapy (4). As to molecular genetic changes, many pancreatic adenocarcinomas harbor mutations in the ras oncogene and to a lesser extend in the tumor suppressor genes p53 and DPC4/SMAD4 (5). Other cell cycle relevant molecules that may be altered include p16, p21 (6), and others. The plethora of changes suggests a sequence of molecular events; however, in contrast to colorectal cancer, the step-by-step evolution of these mutations or deletions remains to be elucidated (7).

The tumor is made up of the malignant cells and a dense stroma (8). Within the tumor tissue, immunocompetent cells are interspersed, among them monocytes and lymphocytes (9). The function of these infiltrating mononuclear cells is under debate. To some extent, their presence reflects a peritumoral inflammation or even mild pancreatitis (9).

Apoptosis is a cell suicide mechanism that plays a central role in development and homeostasis of multicellular organisms. Cells die by apoptosis in the developing embryo and in adult animals during tissue turnover, or at the end of an immune response (10,11). Apoptosis is also the mechanism by which tumor cells die when treated with therapeutic agents (12–16). Hope was increasing

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that this observation could be exploited to select for novel therapeutic strategies targeting apoptosis-inducing receptors in highly aggressive tumors. This strategy showed promise in some cases, such as gliomas, colon, and breast cancer cells, and certain types of melanomas (17–21). However, recently a novel mechanism of immune evasion by tumor cells has been described, namely "the tumor counter-attack model" (22). In this model, tumor cells kill activated lymphocytes through functional expression of the apoptosis-inducing ligand, Fas ligand (FasL) (22,23). This raises the possibility that apoptosis pathways, such as FasL/Fas (CD95) and DR4, DR5/TRAIL, could be used by tumors to evade the immune system. Indeed, many publications showed that malignant tumors express high levels of functional FasL and TRAIL and are resistant to apoptosis induced by these molecules (24–26). Others do not express the ligands but, nevertheless, are resistant to cell death, suggesting that tumors select for highly aggressive clones through these pathways as well (27).

FasL and TRAIL/tumor necrosis factor-related apoptosis inducing ligand are two highly homologous tumor necrosis factor gene superfamily members with the ability to induce apoptosis in susceptible cells through interaction with their membrane receptors Fas and DR4, DR5 respectively (28,29). These receptors have a homologous 80 amino acid domain, called the death domain, that is responsible for the initiation of the intracellular signaling cascade leading to cell death. Trimerization of the receptors through the interaction with their ligands lead to the recruitment and activation of fas-associated death domain, an adaptor molecule that recruits and activates caspase 8, an interleukin 1B-converting enzyme-related protease. Consecutive recruitment of caspase 3 and other proteases leads to the disorganization of plasma membranes, blebbing, DNA and cellular protein fragmentation, and cell death (30,31). Both death pathways (FasL and TRAIL) are inhibited by bcl-2–related proteins, bcl-2, and bcl-xL, as well as by fas-like IL-1 converting enzyme (FLICE)-like inhibitory protein and are facilitated by Bax (31). Additionally, TRAIL and FasL interact with receptors that lack the death domain, the decoy receptors DcR1 DcR2 for TRAIL and DcR3 for FasL (32). Identification of these decoy receptors adds further complexity to the regulation of TRAIL/FasL pathways. Recent experiments with animal models suggest that tumor cells are eliminated in vivo after treatment with TRAIL, with no apparent side effects on normal tissues (19). Indeed, TRAIL induces apoptosis in a wide variety of malignant cell lines but does not show cytotoxicity to normal cells. This suggests that TRAIL is a safe agent for cancer therapy, unlike earlier studies with anti-Fas antibody therapy that led to death of the animals (33).

This study therefore addressed the following questions: (i) Do pancreatic adenocarcinoma cells express intact apoptosis machineries?; (ii) Are they susceptible to TRAIL mediated apoptosis?; and (iii) Could treatment with recombinant TRAIL improve the cytotoxic effectiveness of commonly used therapeutic agents such as gemcitabine?

MATERIALS AND METHODS

Cell lines and reagents

A panel of well-characterized human pancreatic adenocarcinoma cell lines was used: AsPC-1, BxPC-3, and PANC-1 cells (all from American type culture collection [ATCC]), as well as PancTu and PaCa-44 cells (34,35). Jurkat and HeLa cells (ATCC) were used as controls (30). All cells were cultivated in DMEM/Glutamax I supplemented with 10% heat-inactivated fetal calf serum and antibiotics (100 units/mL penicillin; 50 μg/mL streptomycin-G] (Gibco/BRL, Karlsruhe, Germany).

RT/PCR

Cells were trypsinized, washed twice with phosphate buffered saline (PBS) and total RNA was prepared using Qiagen RNA extraction kit. cDNA was prepared following standard protocols. PCR was performed using TFL polymerase and a standard buffer supplied by the manufacturer (BioZym, Hamburg, Germany). Conditions were: an initial denaturation step for 2 minutes at 94°C then 30 seconds at 94°C, 30 seconds at 60°C, and 50 seconds at 72°C for 30 cycles followed by an elongation step for 7 minutes at 72°C. The following primers were used for PCR: β-actin upstream: 5′-GCC GCC AGC TCA CCA TGG-3′ and downstream: 5′-CTC CTC GGG AGC CAC ACG-3′; Fas upstream: 5′-GCA ACA CCA AGT GCA AAG AGG-3′ and downstream: 5′-GTC ACT AGT AAT GTC CTT GAG G-3′; TRAIL upstream: 5′-CAG GAT CAT GGC TAT GAT GG-3′ and downstream: 5′-GAC CTC TTT CTC TCA CTA GG-3′; FasL upstream: 5′-CCA GAG AGA GCT CAG ATA CGT TGA C-3′ and downstream 5′-ATG TTT CAG CTC TTC CAC CTA CAG A-3′; DcR3 upstream: 5′-TGC TCC AGC AAG GAC CAT GA-3′ and downstream: 5′-GTG CTG CTG GCT GAG AAG GT-3′; DR4 upstream: 5′-ACA CAG CAA TGG GAA CAT AGC-3′ and downstream: 5′-TTGTGAGCATTGTCCT-CAGC-3′ (18); DR5 upstream: 5′-GGG AGC CGC TCA TGA GGA AGT TGG-3′ and downstream: 5′-GGC AAG TCT CTC TCC CAG CGT CTC-3′ (18); DcR1

upstream: 5′-GAA GAA TTT GGT GCC AAT GCC ACT G-3′ and downstream: 5′-CTC TTG GAC TTG GCT GGG AGA TGT G-3′ (25); DcR2 upstream: 5′-CTT TTC CCG CGG CGT TCA TGT CCT TC-3′ and downstream: 5′-GTT TCT TCC AGG CTG CTT CCC TTT GTA G-3′ (25). PCR fragments were separated in 2% agarose gels (Nusieve/GMC) and visualized by ethidium bromide. β-actin was used as an internal control for cDNA input and a water control was performed in each run to control for cross contaminations.

FasL Real-Time (TaqMan) RT-PCR

The primer pair and probe were designed using the Primer Express 1.0 program (PE Applied Biosystems, Foster City, CA, U.S.A.). They have the following sequences: TaqMan probe 5′-TCC AAC TCA AGG TCC ATG CCT CTG G-3′, forward primer 5′-AAA GTG GCC CAT TTA ACA GGC-3′, and reverse primer 5′-AAA GCA GGA CAA TTC CAT AGG TG-3′; all were obtained from Applied Biosystems GmbH (Weiterstadt, Germany). The primers yielded RT-PCR products of 82 bp (FasL). Direct sequencing of the PCR product was performed to avoid the possibility of PCR artifacts. For calibration of the FasL TaqMan-assays, two RNA standards were generated by using an in vitro T7-Polymerase transcription system (RiboMAX Large Scale RNA Production System; Promega, Madison, WI, U.S.A.). Using the TaqMan 5′- and 3′-primers, a preparative standard PCR reaction was performed to produce a FasL-specific fragment. The fragment was then cloned into a SmaI linearized pBLUESCRIPT KS vector (Stratagene, La Jolla, CA, U.S.A.). The resulting in vitro transcripts were used to prepare stock dilution series, in yeast tRNA, over eight logs from 10^9 to 10^2 specific RNA molecules. The TaqMan EZ RT-PCR Kit (PE Applied Biosystems) was used for reverse transcription and amplification of both targets and standards. Production of cDNA and PCR-amplification was carried out in a single-tube, single-enzyme system without the addition of subsequent enzymes or buffers (36). All RT-PCR reactions were performed in duplicate with a final volume of 25 μL. The reaction conditions were 2 minutes at 50°C, 30 minutes at 60°C, 5 minutes at 95°C, 35 cycles with 20 seconds at 94°C and 1 minute at 60°C. The quantification of FasL RNA standards was linear over eight logs and the assay can measure as little as 100 copies of FasL mRNA copies per tube (36). The threshold cycle values decreased linearly with increasing target quantity. In the experiment, the correlation coefficient was 0.995.

Western blotting

Cells were preincubated with 5 ng/mL Phorbol 12-myristate 13-acetate (PMA, Sigma, St. Louis, MO, U.S.A.) for 24 hours, followed by 200 ng/mL TRAIL (R&D) for 8 hours (DcR1, CPP32, FADD, TRAIL, Bax, bcl-2, bcl-xL, DR4). Proteins were separated by SDS-PAGE and transferred to a polyvinylidene difluoride membrane (Roche), as described previously. Equal amounts of proteins (25 μg) were loaded on gels. Blot membranes were blocked for 3 hours at 37°C in Tris-buffered saline (TBS, 10 mM Tris, 10 mM NaCl) containing 5% skim milk and probed with the respective antibodies (16 hours at 4°C). The following antibodies were used in a dilution of 1:1,000: Bax (Santa Cruz [sc], sc-493), bcl-2 (sc-509), bcl-xL (sc-1690), DcR1 (sc-7193), DR4 (sc-7863), caspase 3 (CPP32; sc-1226), FADD (sc-1172), TRAIL (sc-6079), and Fas (C20). Secondary antibodies (all from Dako, 1:5,000 for 1 hour at room temperature) were mouse-anti-goat Ig, rabbit-anti-mouse Ig, and porcine-anti-rabbit Ig-AP. Detection was performed by chemiluminescence (37).

FACS analysis

For flow cytometric analysis of Fas and FasL expression cells were detached with 5-mM EDTA solution and washed twice with PBS. Cells (10^5) were incubated with the PE-conjugated anti-Fas antibody UB2 or with an isotype-matched control mAb (Immunotech, Hamburg, Germany) for 30 minutes at 4°C. Thereafter, the cells were washed with ice-cold PBS. For determination of FasL expression, nonpermeabilized and permeabilized cells were stained with anti-human FasL (clone NOK-1) or an isotype control (Pharmingen, Hamburg, Germany), as described previously, followed by the incubation with 100 μL FITC-conjugated secondary antibody (Sigma) for 30 minutes at 4°C. For permeabilization, cells were treated as previously described (38). Determination of Fas and FasL expression was performed in a FACScan flow cytometer (Becton Dickinson, Franklin Lakes, NJ) using FACScan Research software and measuring 10,000 cells of each sample.

Induction of apoptosis and cytotoxicity assays

The day before treatment, 2×10^6 pancreatic tumor cells were seeded in 60-mm Petri dishes and cultivated in DMEM/Glutamax I with 10% FCS. The cells were washed three times with PBS during the next day. The treatment groups received complete medium (as described above), supplemented with 200 ng/mL TRAIL (R&D) and/or 200 ng/mL Gemcitabine (Lilly GmbH, Hamburg, Germany) for 24 hours. The cells were then harvested. Before trypsination, the supernatant was collected and spun down (1,000 rpm for 5 minutes). The adherent cells were trypsinized and washed in PBS. Both cell fractions were combined and analyzed further via

FACS and Western blotting. Cell viability and apoptosis were determined by propidium iodide staining (15). Fluorescence intensity was measured by FACScan flow cytometer (Becton Dickenson), a method that is widely used to measure the percentage of apoptotic cells (39).

RESULTS

Expression of Fas L and its receptors Fas and DcR3 in human pancreatic adenocarcinoma cell lines

The expression of FasL, its receptor Fas, and decoy receptor DcR3 were assessed by RT/PCR (Fig. 1). All five tumor cell lines expressed the receptors Fas and DcR3. Expression of Fas was confirmed at the protein level by FACS analysis and Western blotting (Fig. 2A, B, E). Even though all cells expressed the Fas protein clearly, levels of expression were variable. Contrary to what has been shown for other tumors, and by others for pancreatic tumor cell lines, we could not detect FasL expression by RT/PCR or by FACS analysis of intact or permeabilized cells (Fig. 2C, D). Only one cell line, AsPC1, showed weak intracellular staining, after permeabilization, using the NOK1 antibody. We could not confirm these results by Western blotting because the specificities of the commercially available anti-FasL antibodies have been called into question in recent reports and, as such, are not reliable (40). Because of this controversy, we have established an accurate and very sensitive quantitative RT/PCR TaqMan assay for Fas ligand (Fig. 2f). The assay is capable of detecting 1,000 molecules FasL mRNA in 1 μg of total RNA (36). The finding confirmed that no FasL could be detected in these cell lines. All five cell lines expressed fewer than 1,000 RNA molecules/1 μg RNA as compared to 2,500 molecules in normal pancreas, 9,600 molecules in nonactivated Jurkat cells (Fig. 2F), and 410,000 molecules in spleen (data not shown).

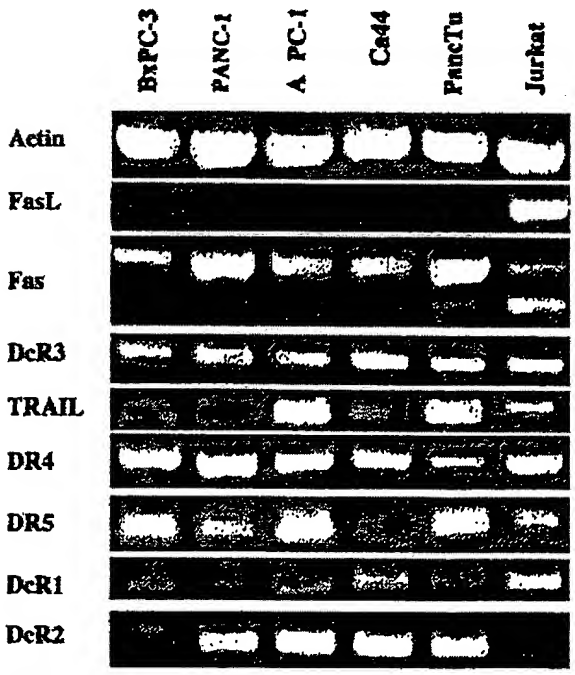

FIG. 1. RT/PCR of apoptotic receptors and ligands in pancreatic carcinoma cell lines. The assay was performed as described in materials and methods. Lanes represent cell lines: BxPc-3, Panc-1, AsPC-1, PaCa 44, PancTu, and Jurkat, respectively.

Expression of TRAIL and its receptors DR4, DR5, DcR1, and DcR2

The expression of TRAIL and its receptors DR4 and DR5, and decoy receptors DcR1 and DcR2, was assessed by RT/PCR (Fig. 1). All cell lines expressed these molecules at different levels. We confirmed the expression of TRAIL and DR4 at the protein level by Western blotting (Fig. 3). However, DcR1 was not detected by Western blotting. Only TRAIL-treated Jurkat cells expressed high levels of DcR1 protein.

Expression of apoptosis-signaling molecules

We then characterized the apoptosis mechanism in pancreatic cell lines by Western blot (i.e., the intracellular-signaling molecules FADD and caspase 3 (Fig. 3A) and the anti- and pro-apoptotic molecules of the bcl-2 gene family bcl-2, bcl-xL, and Bax (Fig. 3B). Both molecules (Bax and bcl-2) were known to be overexpressed in malignant pancreatic tissues (41). All cell lines expressed FADD and caspase 3 either constitutively (PaCa44, Panc-1) or after treatment with TRAIL, (BxPC-3) (Fig. 3A). Bax, bcl-2, and bcl-xL proteins were detected in all cell lines, confirming earlier results and indicating a possible role in malignancy.

Pancreatic carcinomas susceptibility to TRAIL-induced apoptosis is not augmented by gemcitabine

Because all cell lines expressed many molecules required for cell death, we examined their susceptibility to TRAIL-mediated apoptosis. Cell lines were treated with recombinant soluble TRAIL, gemcitabine, or a combination of both. Two cell lines (BxPC-3 and Panc-1) were susceptible to apoptosis because nearly 50% and 60% of treated cells died after 24 hours of treatment with TRAIL (Fig. 4A). The treatment of cells with gemcitabine alone or in combination with TRAIL did not affect the percentage of apoptotic cells in all cell lines tested (Fig. 4B).

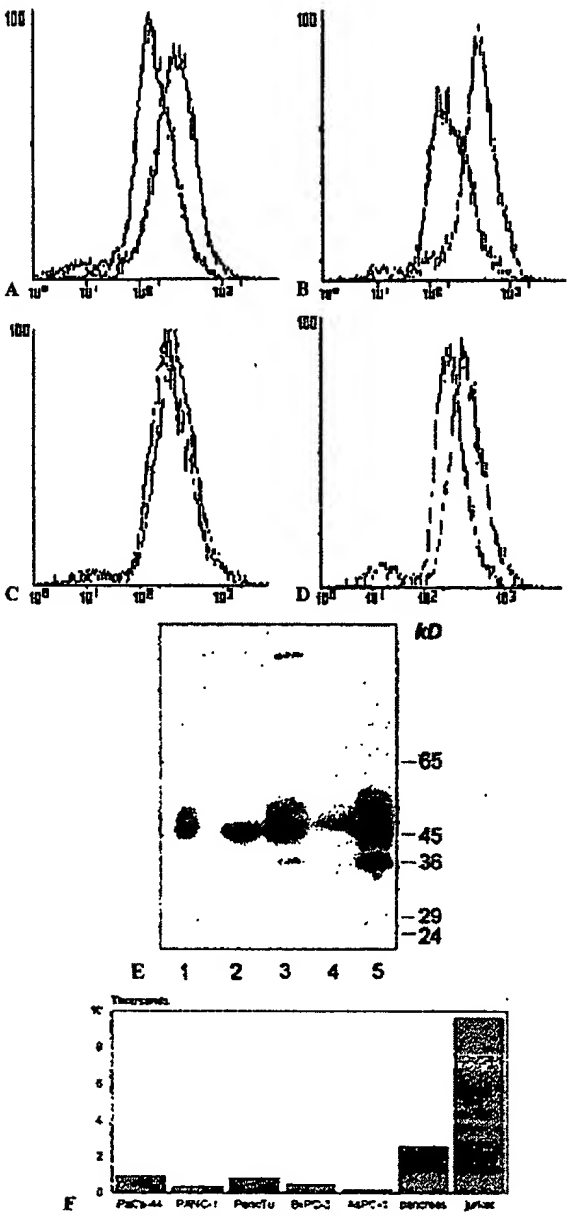

FIG. 2. Expression of Fas and FasL in pancreatic carcinoma cell lines. FACS analysis of Fas surface expression by AsPC1 (A) and PancTu (B) was performed using PE-conjugated anti-Fas antibody UB2 or an isotype matched control mAb. Staining of FasL in nonpermeabilized (C) or permeabilized (D) AsPC1 cells was performed using the mAb NOK-1 or an isotype matched mAb control. (E) Western blotting of whole-cell lysates was performed with rabbit anti-Fas polyclonal antibodies (C20, SantaCruz, CA, U.S.A.). Lanes 1–5 represent cell lines PaCa 44, AsPC-1, PancTu, BxPC-3, and Panc-1, respectively. Detection was performed by chemiluminescence. Molecular weight markers are indicated. (F) Expression of FasL mRNA in pancreatic cell lines, pancreas, and nonactivated Jurkat cells was measured by real-time (TaqMan) RT/PCR. Numbers of specific mRNA molecules per µg RNA (Y-axis) are shown.

The more aggressive cell lines (PaCa44 and AsPC-1) were completely resistant to apoptosis.

DISCUSSION

Our results suggest that tumor cells have exploited the apoptosis pathways, FASL/TRAIL, and evolved elaborate mechanisms to evade the immune system and select for highly aggressive clones. Indeed, expression of apoptosis-inducing ligands, such as TRAIL and FasL, could be beneficial to tumors in three different ways. First, induction of cell death in infiltrating T cells (the tumor-counterattack model) (27). Second, it could facilitate metastasis by inducing cell death of normal tissues. This is plausible because many tissues or cell types express Fas and DR4/DR5 and are susceptible to apoptosis. This mechanism was also suggested for the propagation of metastasis in the liver (42). The third possibility is that it eliminates low aggressive clones. Indeed, in similar aggressive tumors, namely melanomas, the most aggressive metastasising clones are those with the highest rate of turnover, cell death, and they express the highest levels of Fas Ligand (43) (M. Kunz, personal communica-

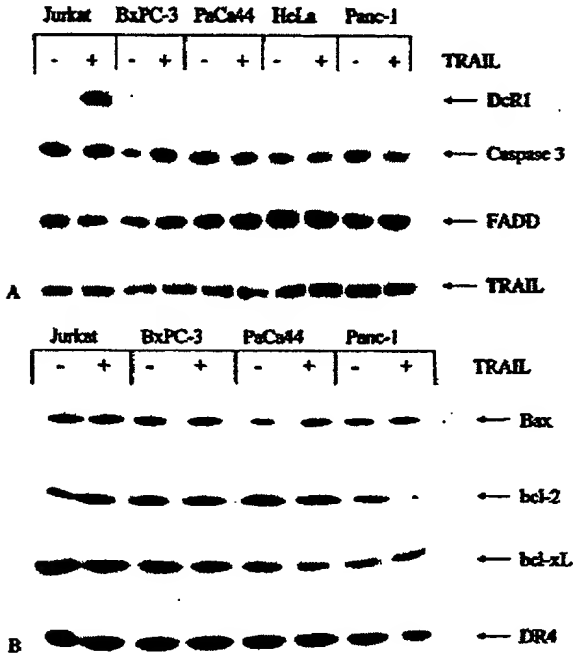

FIG. 3. Expression of TRAIL, its receptors, and other signaling molecules in pancreatic tumor cell lines. Western blot analysis of whole cell lysates for DcR1, caspase 3, FADD, and TRAIL (A) and Bax, bcl-2, bcl-xL, and DR4 (B) in cells before or after 8 hours of TRAIL treatment (200 ng/mL).

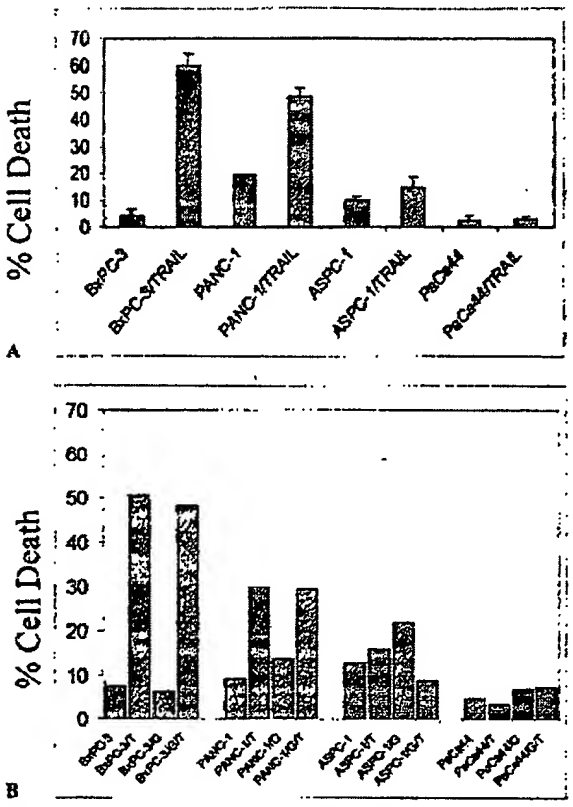

FIG. 4. Sensitivity of pancreatic adenocarcinoma cells to TRAIL induced apoptosis. (A) Cells were treated with 200 ng/mL of TRAIL. Percentage of cell death in cells treated with TRAIL or grown in cell culture alone are shown. Data are expressed as means of dead cells + SD (n = 3). (B) Addition of gemcitabine (/G) did not affect cell death alone or in combination with TRAIL (/T).

tions, 2000). Recently, we have shown that survival, metastasis, and recurrence rates among breast cancer patients correlate strongly with high levels of Fas ligand expression (36). Unlike other tumors, such as colon and breast carcinomas in which the expression of the Fas receptor is down-regulated in aggressive tumors (27), pancreatic carcinomas seem to express high levels of nonfunctional receptor (37,38,44). This phenomenon could be beneficial to the tumor because interaction between Fas expressed on tumor cells and FasL on activated CD4+ T cells leads to cell cycle arrest of lymphocytes (45). Approximately 45% of tumor-infiltrating T cells are CD4+, and eliminating these cells is essential for tumor survival (9).

Members of the bcl-2 gene family (Bax and bcl-2) seem to be expressed by primary pancreatic carcinomas (14), but only Bax correlated with a favorable diagnosis (41), as observed for colorectal cancer patients with liver metastasis (46). We found that bcl-xL is also highly expressed in pancreatic carcinoma cell lines. This could reflect another mechanism of tumor survival similar to those of other hepatic and gastrointestinal and breast tumors (47–49).

The partial success in inducing apoptosis in pancreatic cell lines (two of four tested) is encouraging in that the promising TRAIL immunotherapy approach could be extended to pancreatic carcinomas.

Gemcitabine is the quasi-standard chemotherapy for pancreatic carcinoma (50). We therefore wanted to investigate the effect of this cytostatic drug on the apoptotic mechanisms in pancreatic carcinoma. The failure of gemcitabine to augment TRAIL-induced cell death, despite the fact that chemotherapeutic agents enhance the apoptosis in other tumors, should encourage studies into other combinations of TRAIL/drugs (17,51,52). Other cytokines, such as TNF-α, did not influence apoptosis in Panc-1 cells but TRAIL, as shown in our experiments, induced cell death (53). In a variety of carcinoma cells, classical cytostatic drugs, such as etoposide and vinblastin as well as metalloproteinase inhibitors (54), induce apoptosis by upregulating Fas (CD95) and its ligand (13,16). Interestingly, the PaCa-44 cells line resistant in vitro underwent apoptosis in a nude mouse model using local conversion of ifosfamide (15), suggesting that a pathway, independent of FasL and TRAIL, could be involved. The CD95L upregulation is, at least in part, facilitated by reactive oxygen intermediates (55).

The resistance of cell lines derived from highly aggressive tumors (e.g. PancTu) raises the possibility that TRAIL therapy could be beneficial, but could accelerate the selection process for highly aggressive cells by eliminating low aggressive cells, thus allowing clones that are apoptosis-resistant the chance to expand and metastasise (42). It is tempting to speculate that the differential susceptibility to apoptosis in pancreatic adenocarcinomas could be due to different patterns of mutations in p53 and ras (56–62). However, published data do not allow us to draw a clear conclusion at the present time..At least p53 was shown to be involved in both TRAIL and FasL-mediated apoptosis (16,63–65).

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Anti-human TRAIL R1/DR4/TNFRSF10A Antibody

ORDERING INFORMATION

Catalog Number: AF347

Lot Number: BWF01

Size: 100 µg

Formulation: 0.2 µm filtered solution in PBS

Storage: -20° C

Reconstitution: sterile PBS

Specificity: human TRAIL R1

Immunogen: *Sf* 21-derived rhTRAIL R1 extracellular domain

Ig class: human TRAIL R1 extracellular domain specific goat IgG

Applications: Neutralization of bioactivity
ELISA
Western blot
Immunohistochemistry

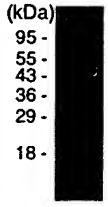

Figure 1: Immunoblots of SDS extracts from 2 x 10^5 TF1 cells using 1.0 µg/mL of goat anti-TRAIL R1.

Preparation
Produced in goats immunized with purified, insect cell line *Sf* 21-derived, recombinant human TRAIL R1 (rhTRAIL R1) extracellular domain. TRAIL R1 specific IgG was purified by human TRAIL R1 affinity chromatography.

Formulation
Lyophilized from a 0.2 µm filtered solution in phosphate-buffered saline (PBS).

Endotoxin Level
< 10 ng per 1 mg of the antibody as determined by the LAL method.

Reconstitution
Reconstitute with sterile PBS. If 1 mL of PBS is used, the antibody concentration will be 0.1 mg/mL.

Storage
Lyophilized samples are stable for greater than six months when held at -20° C to -70° C. Upon reconstitution, the antibody can be stored at 2° - 4° C for at least 1 month without detectable loss of activity. Reconstituted antibody can also be aliquotted and stored frozen at -20° C to -70° C for at least six months without detectable loss of activity. **Avoid repeated freeze-thaw cycles.**

Specificity
This antibody has been selected for its ability to recognize human TRAIL R1 in western blots and direct ELISAs. In western blots, this antibody shows approximately 10% cross-reactivity with rhTRAIL R2 and no cross-reactivity with rhTRAIL R3 and rhTRAIL R4.

Neutralization of Human Soluble TRAIL R1
The exact concentration of antibody required to neutralize soluble TRAIL R1 is dependent on the cytokine concentration, cell type, growth conditions and the type of activity studied. To provide a guideline, R&D Systems has determined the neutralization dose for this antibody under a specific set of conditions. The **Neutralization Dose$_{50}$ (ND_{50})** for this antibody is defined as that concentration of antibody required to yield one-half maximal inhibition of the cytokine activity on a responsive cell line, when that cytokine is present at a concentration just high enough to elicit a maximum response.

The ND_{50} for this lot of anti-human TRAIL R1 antibody was determined to be approximately 0.02 - 0.055 µg/mL in the presence of 10 ng/mL of rhTRAIL R1, using the TRAIL-sensitive mouse L929 cytolytic assay. The specific conditions are described in the figure legends.

Additional Applications
ELISA - This antibody can be used at 0.5 - 1.0 µg/mL with the appropriate secondary reagents to detect human TRAIL R1. The detection limit for rhTRAIL R1 is approximately 0.06 ng/well.

Western blot - This antibody can be used at 0.1 - 1 µg/mL with the appropriate secondary reagents to detect human TRAIL R1. The detection limit for rhTRAIL R1 is approximately 2 ng/lane and 25 ng/lane under non-reducing and reducing conditions, respectively. An immunoblot of SDS extracts from 2 x 10^5 TF1 cells is shown in Figure 1. Extracts were electrophoresed on 12% gels and detection was by ECL procedure (Amersham). A 15 second exposure is shown.

Immunohistochemistry - This antibody will detect TRAIL R1 in paraffin-embedded tissue sections. The working dilution after antigen retrieval is 10 µg/mL. It is recommmended to use R&D Systems' antigen retrieval reagents. For chromogenic detection of labeling, it is recommended to use R&D Systems' Cell and Tissue Staining kits (CTS Series).

Optimal dilutions should be determined by each laboratory for each application.

For immunohistochemistry images, refer to our website at http://www.rndsystems.com/asp/c_immunohistochemistry_add.asp

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Figure 1

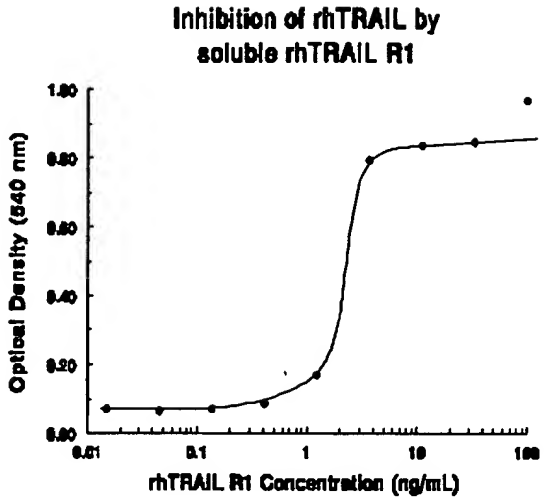
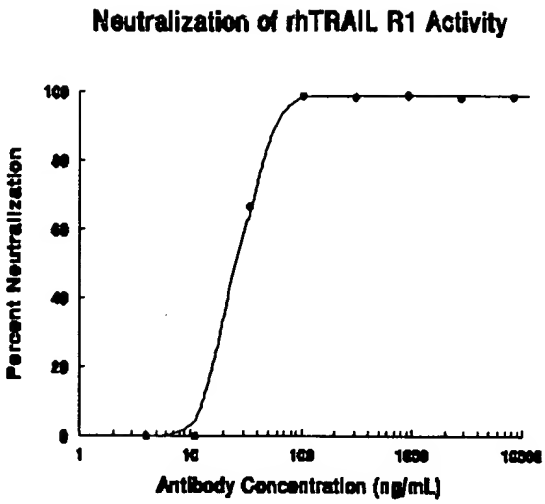


Figure 2



Typical Data

Figure 1

Human soluble TRAIL R1 inhibits TRAIL-induced mouse L929 cell lysis in a dose-dependent manner. The ED_{50} for this effect is typically 1 - 3 ng/mL, in the presence of 20 ng/mL of rhTRAIL and 1 µg/mL of actinomycin D (Matthews, N. and M.L. Neale, 1987, *Lymphokines and Interferons, a practical approach*, Clemens, M.J., Morris, A.G., and A.J.H. Gearing, eds., IRL Press, p. 296).

Figure 2

To evaluate the ability of the antibody to neutralize the biological activity of soluble human TRAIL R1, soluble rhTRAIL R1 was incubated with various concentrations of the antibody in culture media containing actinomycin D for 1 hour at 37° C in a 96 well microplate. Cross-linked rhTRAIL was subsequently added and the mixture was incubated for an additional 30 minutes. Following this preincubation, the assay mixture was transferred to wells in a 96-well microplate containing confluent L929 cells. The assay media, in a total volume of 150 µL/well over a monolayer of confluent L929 cells, containing antibody at the concentrations indicated, cross-linked rhTRAIL at 12 ng/mL, soluble rhTRAIL R1 at 10 ng/mL and actinomycin D at 1 µg/mL, was incubated for 24 hours at 37° C in a 5% CO_2 humidified incubator. At the end of the incubation period, the media was removed, the cells were fixed with 5% formaldehyde and then stained with crystal violet. The stain was subsequently dissolved in 100 µL of 33% acetic acid and the optical density read in a microplate reader set at 540 nm. As shown in Figure 2, the ND_{50} for this lot of antibody is approximately 0.02 - 0.055 µg/mL.

EXHIBIT D

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FADD/MORT1 and Caspase-8 Are Recruited to TRAIL Receptors 1 and 2 and Are Essential for Apoptosis Mediated by TRAIL Receptor 2

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Summary

Apoptosis induced by tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL/APO-2L) has been shown to exert important functions during various immunological processes. The involvement of the death adaptor proteins FADD/MORT1, TRADD, and RIP and the apoptosis-initiating caspases-8 and -10 in death signaling by the two death-inducing TRAIL receptors 1 and 2 (TRAIL-R1 and TRAIL-R2) are controversial. Analysis of the *native* TRAIL death-inducing signaling complex (DISC) revealed ligand-dependent recruitment of FADD/MORT1 and caspase-8. Differential precipitation of ligand-stimulated TRAIL receptors demonstrated that FADD/MORT1 and caspase-8 were recruited to TRAIL-R1 and TRAIL-R2 independently of each other. FADD/MORT1- and caspase-8-deficient Jurkat cells expressing only TRAIL-R2 were resistant to TRAIL-induced apoptosis. Thus, FADD/MORT1 and caspase-8 are essential for apoptosis induction via TRAIL-R2.

Introduction

Apoptosis is an essential process during the development of the immune system and for the maintenance of T and B cell homeostasis. Two apoptosis-inducing members of the tumor necrosis factor (TNF) family, TNF and CD95 ligand (CD95L/FasL/APO-1L), are involved in various immunological processes. These include inflammation, activation-induced T and B cell death, immune privilege, tumor evasion from the immune system, autoimmunity, and AIDS (Nagata, 1997; Krammer, 1999; Wallach et al., 1999). In addition, TNF and CD95L have been shown to kill various tumor cell lines in vitro. TNF and CD95L induce apoptosis upon binding to their cognate receptors capable of transmitting a caspase-activating signal due to the presence of a cytoplasmic death domain (DD) (Krammer, 1999; Peter et al., 1999).

TNF-related apoptosis-inducing ligand (TRAIL/APO-2L) was identified by sequence homology to CD95L and TNF (Wiley et al., 1995; Pitti et al., 1996). Interestingly, TRAIL induced apoptosis in about 60% of tumor cell lines, while most normal cells were resistant. Further, systemic administration of TRAIL/APO-2L suppressed tumor growth in SCID mice and nonhuman primates without being toxic to normal tissue, and TRAIL/APO-2L and chemotherapeutic drugs synergistically suppressed tumor growth in SCID mice (Ashkenazi et al., 1999; Gliniak and Le, 1999; Walczak et al., 1999). In addition, TRAIL/APO-2L was used successfully in locoregional treatment of glioblastoma xenografts in athymic mice (Roth et al., 1999). Thus, TRAIL may serve as a novel treatment for cancer.

The functional expression of TRAIL was recently discovered on the surface of different cells of the immune system previously known to induce apoptosis in target cells by an unidentified mechanism. Among them are type II interferon (IFN-γ)–stimulated monocytes (Griffith et al., 1999b), type I IFN- (IFN-α and IFN-β) or TCR-stimulated T cells (Kayagaki et al., 1999a; Musgrave et al., 1999), nonstimulated $CD4^+$ T cells (Mariani and Krammer, 1998; Thomas and Hersey, 1998; Kayagaki et al., 1999b; Martinez-Lorenzo et al., 1999), IFN-α and IFN-γ-stimulated as well as measles virus-infected dendritic cells (DC) (Fanger et al., 1999; Vidalain et al., 2000), and natural killer (NK) cells (Zamai et al., 1998; Johnsen et al., 1999; Kashii et al., 1999; Kayagaki et al., 1999c).

TRAIL interacts with five distinct receptors: TRAIL-R1 (DR4) (Pan et al., 1997b), TRAIL-R2 (DR5/TRICK2/KILLER) (MacFarlane et al., 1997; Pan et al., 1997a; Schneider et al., 1997a; Screaton et al., 1997; Sheridan et al., 1997; Walczak et al., 1997), TRAIL-R3 (DcR1/TRID/LIT) (Degli-Esposti et al., 1997a; Pan et al., 1997a; Schneider et al., 1997a; Sheridan et al., 1997; Mongkolsapaya et al., 1998), TRAIL-R4 (DcR2/TRUNDD) (Degli-Esposti et al., 1997b; Marsters et al., 1997; Pan et al., 1998), and Osteoprotegerin (OPG) (Emery et al., 1998). TRAIL-R1 and TRAIL-R2 contain an intracellular DD necessary for apoptosis induction upon TRAIL-mediated receptor ligation. In contrast, neither TRAIL-R3 nor TRAIL-R4 can mediate apoptosis due to complete or partial absence of an intracellular DD, respectively. OPG is a soluble receptor reported to bind OPG ligand (OPGL/RANKL/TRANCE/ODF) and TRAIL (Emery et al., 1998).

The biochemical events leading to apoptosis induction via TNF and CD95 have been analyzed in detail. Cross-linking of CD95 leads to the formation of a death-inducing signaling complex (DISC) (Kischkel et al., 1995). The death adaptor protein FADD/MORT1 (Boldin et al., 1995; Chinnaiyan et al., 1995) and the proteolytic enzyme caspase-8 (Boldin et al., 1996; Muzio et al., 1996) are recruited to the CD95 DISC (Kischkel et al., 1995). In a homotypic interaction, the DD of FADD/MORT1 binds to the DD of CD95. The death effector domain (DED) of FADD/MORT1 in turn interacts with the DED of procaspase-8 and thereby recruits this proenzyme to the

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CD95 DISC (Medema et al., 1997). Pro-caspase-8 is proteolytically cleaved and thereby activated at the DISC. Activated caspase-8 then initiates the apoptosis executing caspase cascade (Peter et al., 1999).

TNF induces apoptosis by cross-linking of its DD-containing receptor TNF-R1 (p55). Ligand-induced cross-linking of TNF-R1 leads to the recruitment of TRADD and RIP to the DD of the receptor. FADD/MORT1 is recruited to the TNF-R1 DISC via TRADD. In analogy to the CD95 system, FADD/MORT1 then recruits caspase-8 to the TNF-R1 DISC by homotypic interaction of their respective DEDs resulting in activation of caspase-8 and apoptosis. Signals emanating from RIP and TRADD-recruited TRAF2 result in activation of the NF-κB and Jun kinase pathways, respectively. These gene-inductive events are responsible for the inflammatory processes associated with triggering of TNF-R1 (Wallach et al., 1999).

Given its implication in various immunological processes, it is important to understand the biochemical mechanism of initiation of TRAIL-induced apoptosis. So far, this mechanism was addressed in a number of overexpression studies examining the role of different known apoptosis signaling proteins. In some overexpression systems, dominant-negative FADD/MORT1 (FADD-DN) inhibited TRAIL-induced apoptosis (Chaudhary et al., 1997; Schneider et al., 1997b; Walczak et al., 1997; Wajant et al., 1998). In other studies, FADD-DN overexpression did not prevent TRAIL-induced apoptosis (MacFarlane et al., 1997; Pan et al., 1997a, 1997b; Sheridan et al., 1997). Coimmunoprecipitations of overexpressed cytoplasmic domains of TRAIL-R1 or TRAIL-R2 with FADD/MORT1 supported a role for FADD/MORT1 in TRAIL-induced apoptosis (Chaudhary et al., 1997; Schneider et al., 1997b). Yet, opposite results were obtained in another study, as FADD/MORT1 was not coimmunoprecipitated with overexpressed TRAIL-R2 but only with CD95 (MacFarlane et al., 1997). Murine embryonic fibroblasts (MEF) from FADD/MORT1-deficient mice underwent apoptosis upon overexpression of human TRAIL-R1 (DR4) (Yeh et al., 1998). Thus, the role of FADD/MORT1 in TRAIL-induced apoptosis is still unclear.

The identity of the caspase(s) involved in the initiation of TRAIL-mediated apoptosis is also controversial. Under native conditions, early cleavage of caspase-8 has been observed following TRAIL stimulation (Griffith et al., 1998; Leverkus et al., 2000). Yet, overexpression studies with dominant-negative forms of caspase-8 and -10 either suggested caspase-10 alone (Pan et al., 1997a) or a combination of caspase-8 and -10 (MacFarlane et al., 1997) as initiator caspase(s) during TRAIL-induced apoptosis. In addition, TRAIL resistance of mature DC and activated peripheral T cells from ALPS II patients was suggested to be due to mutated nonfunctional caspase-10 and to be causative for the disease (Wang et al., 1999).

To dissect the molecular mechanisms of TRAIL-induced apoptosis initiation, we differentially analyzed the native TRAIL-R1 and TRAIL-R2 signaling complexes induced upon TRAIL stimulation of sensitive target cells. Our data indicate that, independently of each other, both TRAIL-R1 and TRAIL-R2 recruit FADD/MORT1 and caspase-8 in a ligand-dependent fashion. In addition, we show that FADD/MORT1 and caspase-8 are essential for TRAIL-R2-mediated apoptosis.

Results

BL60, BJAB, and CEM Cells Are Susceptible to TRAIL-R1- and TRAIL-R2-Induced Apoptosis, whereas Jurkat Cells Express Only Functional TRAIL-R2

In order to dissect the apoptosis-inducing events immediately following receptor stimulation by TRAIL in lymphoid cells, we chose the human B cell lines BL60 and BJAB and the human T cell lines CEM and Jurkat as model systems. We characterized these cell lines by determination of TRAIL sensitivity, analysis of the time course of caspase-8 activation, and their TRAIL receptor expression profile. We then analyzed the functionality of TRAIL-R1 and TRAIL-R2 signaling separately. All four cell lines were susceptible to LZ-TRAIL-induced apoptosis (Figure 1A). In accordance with studies on non-lymphoid cell lines (Griffith et al., 1998; Leverkus et al., 2000), stimulation of BL60, BJAB, CEM, and Jurkat cells with LZ-TRAIL led to early cleavage and activation of caspase-8 (Figure 1B) prior to cleavage of caspase-3 and PARP (data not shown). This supports an initiating role for caspase-8 during TRAIL-induced apoptosis also in lymphoid cells. While BL60, BJAB, and CEM cells expressed both apoptosis-inducing TRAIL receptors, only TRAIL-R2 was present on the surface of Jurkat cells (Figure 1C, top panels). TRAIL-R3 and TRAIL-R4 were not detectable on the surface of these cell lines (Figure 1C, bottom panels).

We next analyzed whether TRAIL-R1 and/or TRAIL-R2 were functionally expressed. After preincubation with soluble blocking monoclonal antibodies (mAbs) against TRAIL-R1, TRAIL-R2, or a combination thereof, we treated the different cells with LZ-TRAIL (Figure 1D). While the combination of TRAIL-R1- and TRAIL-R2-specific mAbs inhibited TRAIL-induced apoptosis in all four cell lines, preincubation with anti-TRAIL-R2 alone blocked TRAIL-induced apoptosis only in Jurkat cells. Preincubation with blocking anti-TRAIL-R1 alone did not inhibit TRAIL-induced apoptosis in any of the cell lines tested. Thus, BL60, BJAB, and CEM cells are susceptible to apoptosis mediated by ligand-induced cross-linking of either TRAIL-R1 or TRAIL-R2, whereas Jurkat cells can only be killed via TRAIL-induced cross-linking of TRAIL-R2. These data show that these antibodies are suitable for differential analysis of the individual protein complexes formed upon TRAIL-mediated cross-linking of the two different apoptosis-inducing TRAIL receptors under native conditions.

TRAIL-Dependent Recruitment of Caspase-8 and FADD/MORT1 to the TRAIL DISC in BL60 Cells

The active caspase-8 subunit p18 could be detected as early as 15 min after TRAIL stimulation (Figure 1B), suggesting that caspase-8 might be the caspase responsible for initiation of TRAIL-induced apoptosis. The *native* protein complex that initiates TRAIL-induced apoptosis was analyzed for presence of the various signaling proteins that have been suggested to play a role at

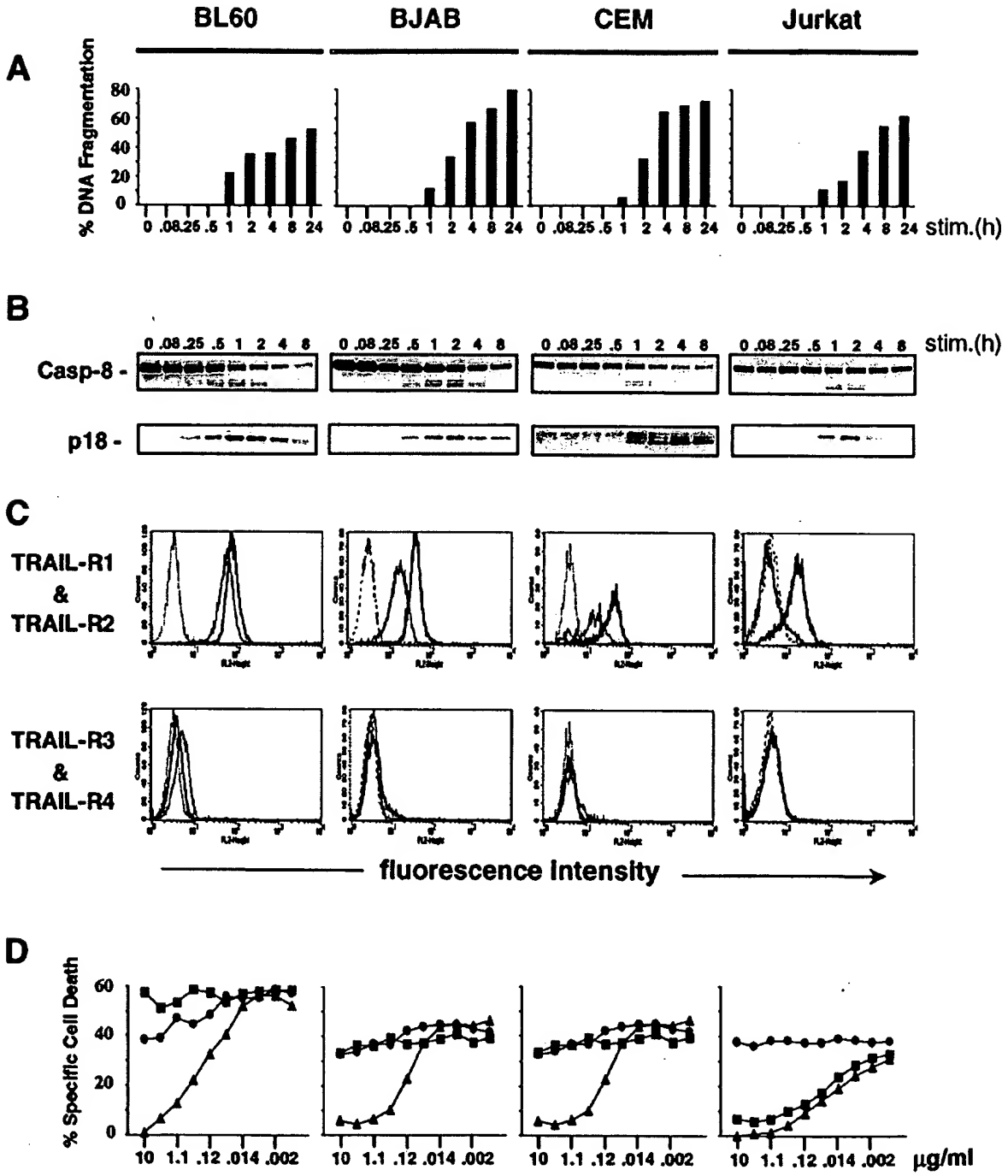

Figure 1. Analysis of Kinetics of TRAIL-Induced Apoptosis, Caspase-8 Cleavage, Receptor Expression Profile, and Functional Expression of TRAIL-R1 and TRAIL-R2 in BL60, BJAB, CEM, and Jurkat Cells

(A and B) Cells were stimulated with 1 µg/ml LZ-TRAIL for the indicated time periods followed by analysis of percentage of cells with subdiploid DNA content (A) and Western blot analysis of caspase-8 (p55/53) and its p18 cleavage product (B).

(C) FACS analysis of surface expression of TRAIL-R1 (upper panels, solid lines); TRAIL-R2 (upper panels, solid bold lines); TRAIL-R3 (lower panels, solid lines); and TRAIL-R4 (lower panels, solid bold lines) as compared to an isotype-matched control mIgG1 mAb (dashed lines).

(D) Identification of TRAIL-R1- and TRAIL-R2-inhibiting mAbs. Cells were treated with 100 ng/ml LZ-TRAIL after 30 min preincubation with or without blocking TRAIL-R1- and/or TRAIL-R2-specific mAbs at the indicated concentrations. The percentage of cell death was determined by FSC/SSC analysis and plotted against the concentration of mAbs specific for TRAIL-R1 (closed circle), TRAIL-R2 (closed square), or both apoptosis-inducing TRAIL receptors (closed triangle). Percentage of specific cell death was calculated as follows: 100 × (% experimental cell death − % spontaneous cell death)/(100 − % spontaneous cell death). Spontaneous cell death was below 10% in all samples. One of four representative experiments is shown.

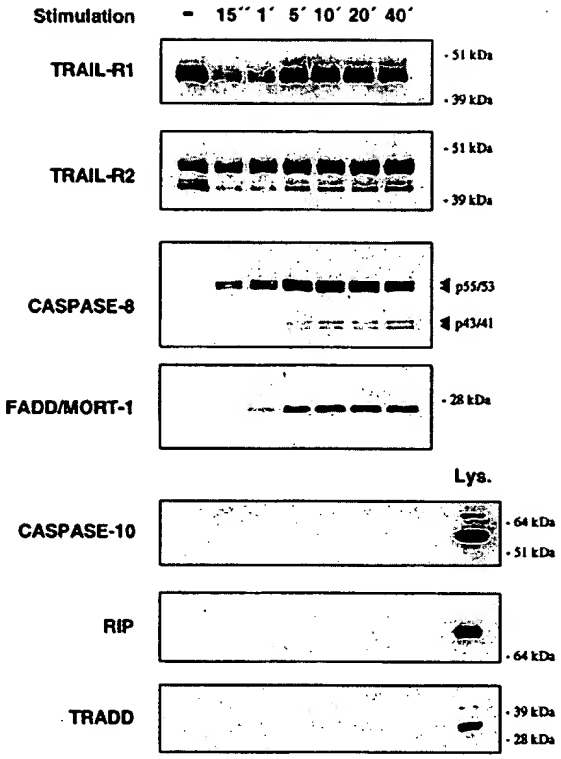

Figure 2. Kinetics of DISC Assembly in BL60 Cells

BL60 cells were either stimulated with 1 μg/ml biotinylated LZ-TRAIL (Bio-LZ-TRAIL) for the indicated time periods or left untreated (−) before cell lysis. Bio-LZ-TRAIL (1 μg/ml) was added to the lysates in the untreated control. Bio-LZ-TRAIL-bound protein complexes were analyzed by SDS-PAGE and Western blot following affinity precipitation with Streptavidin beads. Lysates prepared from BL60 cells (Lys.) are shown as positive controls for caspase-10, RIP, and TRADD antibodies. The upper doublet band detected by the TRAIL-R2 antibody corresponds to the two different splicing variants reported (Screaton et al., 1997), while the lower doublet likely represents proteolytically processed forms of the the two TRAIL-R2 variants (Walczak et al., 1997).

the onset of TRAIL-induced apoptosis (Figure 2). BL60 cells were incubated either in the absence or presence of biotinylated LZ-TRAIL (Bio-LZ-TRAIL) for various time periods. Both TRAIL-R1 and TRAIL-R2 were precipitated with Bio-LZ-TRAIL already after 15 s of stimulation. When analyzing the precipitated protein complex for the presence of caspase-8 and FADD/MORT1, we found that both signaling proteins were recruited as early as 15 s to 1 min after stimulation with Bio-LZ-TRAIL. In addition, cleavage fragments of caspase-8 (p43/41), indicative of activation of the proform of this enzyme (p55/53), were detectable within 1–5 min of stimulation. In contrast, caspase-8 and FADD/MORT1 did not associate with nonstimulated TRAIL receptors that were precipitated with Bio-LZ-TRAIL from the lysates of mock-treated cells (Figure 2, left lane). Thus, recruitment of FADD/MORT1 and caspase-8 was dependent on ligand-induced receptor cross-linking. The amount of FADD/MORT1 and caspase-8 recruited to the TRAIL receptors increased in a time-dependent manner reaching maximum levels between 5 min and 20 min of stimulation.

We conclude that TRAIL stimulation leads to the formation of an apoptosis-initiating protein complex, the TRAIL DISC. FADD/MORT1, caspase-8, and the apoptosis-inducing TRAIL receptors, TRAIL-R1 and TRAIL-R2, are integral components of this signaling complex. However, although prominently present in the lysates of BL60 cells, we could not detect caspase-10, RIP, and TRADD associated with the TRAIL DISC at any time point analyzed (Figure 2, bottom panels).

FADD/MORT1 and Caspase-8 Form Part of the TRAIL DISC in Various Lymphoid Cell Lines

We next studied whether FADD/MORT1 and caspase-8 recruitment to the TRAIL DISC can also be observed in other cell lines that express both apoptosis-inducing TRAIL receptors. Therefore, we compared the formation of the TRAIL DISC in BL60 cells with the BJAB and CEM TRAIL DISC. We incubated the cells for 20 min in the presence or absence of Bio-LZ-TRAIL before analyzing the proteins complexed with the biotinylated ligand. TRAIL-R1 and TRAIL-R2 associated with Bio-LZ-TRAIL in BJAB and CEM cells, and both FADD/MORT1 and caspase-8 were recruited to the TRAIL DISC (Figure 3). This association was stimulation dependent, since Bio-LZ-TRAIL added after cell lysis precipitated the nonstimulated receptors but not caspase-8 and FADD/MORT1. Thus, these two signaling proteins, previously identified as integral components of the native CD95 DISC (Kischkel et al., 1995; Muzio et al., 1996), also form part of the native TRAIL DISC in various cell lines.

Jurkat cells only express TRAIL-R2 on their surface (Figure 1C). Thus, Jurkat cells served to test whether cross-linking of TRAIL-R2 in the absence of TRAIL-R1 may also lead to recruitment of FADD/MORT1 and caspase-8. Stimulation of Jurkat cells with Bio-LZ-TRAIL induced recruitment of TRAIL-R2, caspase-8, and FADD/MORT1 in the absence of TRAIL-R1 (Figure 3). Under nonstimulatory conditions, only TRAIL-R2 was bound to TRAIL, indicating that recruitment of FADD/MORT1 and caspase-8 to TRAIL-R2 is dependent on TRAIL-induced cross-linking of TRAIL-R2 on intact Jurkat cells. Thus, homomeric TRAIL-R2 complexes are sufficient for recruitment of FADD/MORT1 and caspase-8 in the absence of TRAIL-R1.

Homomeric TRAIL-R1 and TRAIL-R2 DISCs Recruit FADD/MORT1 and Caspase-8 in BL60 Cells

Since both apoptosis-inducing TRAIL receptors associated with TRAIL upon stimulation, FADD/MORT1 and caspase-8 might have been recruited to homomeric TRAIL-R1, homomeric TRAIL-R2, or heteromeric complexes containing TRAIL-R1 and TRAIL-R2. To differentiate between the proteins associated with homomeric TRAIL-R1 and TRAIL-R2 DISC, respectively, we stimulated the two receptors separately. This was achieved by preincubating BL60 cells in the presence or absence of blocking mAbs against TRAIL-R1 and TRAIL-R2, respectively, before stimulation with Bio-LZ-TRAIL (Figure 4, odd numbered lanes). In the unstimulated controls (Figure 4, even numbered lanes), Bio-LZ-TRAIL added after cell lysis could precipitate not only cell surface-bound but also intracellularly stored TRAIL-R1 and TRAIL-R2, a finding recently described in melanoma

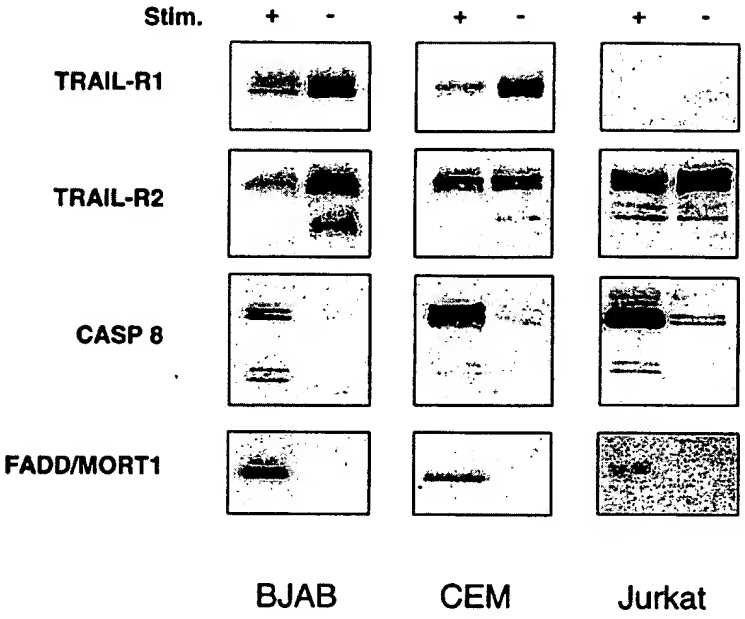

Figure 3. TRAIL DISC Analysis in BJAB, CEM, and Jurkat Cells

BJAB, CEM, and Jurkat cells were left untreated or were treated for 20 min with 1 μg/ml Bio-LZ-TRAIL before cell lysis. Bio-LZ-TRAIL (1 μg/ml) was added to lysates from unstimulated cells for precipitation of unstimulated TRAIL receptors. Bio-LZ-TRAIL-bound protein complexes were analyzed for presence of TRAIL-R1, TRAIL-R2, FADD/MORT1, and caspase-8 by SDS-PAGE and Western blot analysis following affinity precipitation from the lysates with Streptavidin beads.

cells (Zhang et al., 2000). Western blot analysis revealed that in the presence of TRAIL-R2 blocking mAb only TRAIL-R1 was precipitated upon stimulation with Bio-LZ-TRAIL (Figure 4, lane 3, top two panels) and vice versa (Figure 4, lane 5, top two panels). Thus, both homomeric TRAIL-R1 and TRAIL-R2 DISCs were formed upon stimulation with Bio-LZ-TRAIL.

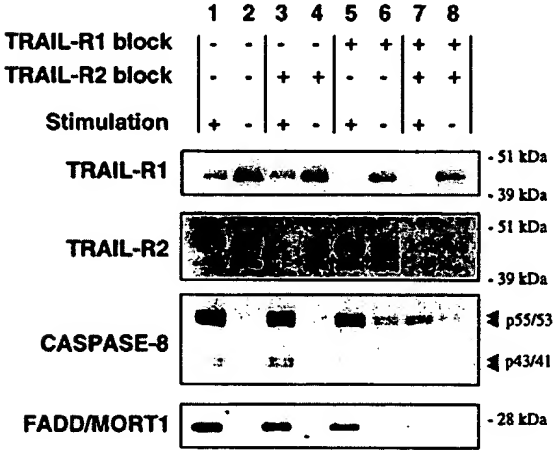

Figure 4. TRAIL Stimulation Induces Recruitment of FADD/MORT1 and Caspase-8 to Homomeric TRAIL-R1 and TRAIL-R2 DISCs in BL60 Cells

After 30 min preincubation with 10 μg/ml blocking TRAIL-R1- and/or TRAIL-R2-specific mAbs, cells were either stimulated with 1 μg/ml Bio-LZ-TRAIL (+) for 20 min or left untreated (−) before cell lysis. Bio-LZ-TRAIL (1 μg/ml) was added to the unstimulated samples for precipitation of unstimulated TRAIL receptors from lysates. Bio-LZ-TRAIL-bound protein complexes were analyzed for presence of TRAIL-R1, TRAIL-R2, FADD/MORT1, and caspase-8 by SDS-PAGE and Western blot analysis following affinity precipitation from the lysates with streptavidin beads. After the longer exposure times necessary to reveal the p43/41 forms of caspase-8, background levels of caspase-8 can be seen in some unstimulated controls.

It is noteworthy that higher amounts of TRAIL-R1 and TRAIL-R2 were detected in the control precipitate when compared to the stimulated condition (Figure 4, lanes 1 and 2). In addition, although antibody blockage before stimulation completely inhibited precipitation of the blocked receptor (Figure 4, lanes 3, 5, and 7), significant amounts of TRAIL-R1 and TRAIL-R2 were precipitated in the unstimulated controls (Figure 4, lanes 4, 6, and 8). The incomplete blockage observed under nonstimulatory conditions indicates that, similar to melanoma cells (Zhang et al., 2000), also BL60 cells contain substantial intracellular amounts of TRAIL-R1 and, to a lesser extent, TRAIL-R2.

Stimulated homomeric TRAIL-R1 and TRAIL-R2 recruited pro-caspase-8 (p55/53), cleavage intermediates of caspase-8 (p43/41), and FADD/MORT1 (Figure 4, lanes 3 and 5). This recruitment was dependent upon ligand stimulation of TRAIL receptors on intact cells since caspase-8 and FADD/MORT1 were not bound to unstimulated TRAIL receptors (Figure 4, lanes 2, 4, 6, and 8). In addition, no other FADD/MORT1 and caspase-8 binding TRAIL receptors were present on BL60 cells as concomitant blockage of TRAIL-R1 and TRAIL-R2 resulted in reduction of ligand-induced precipitation of caspase-8 and FADD/MORT1 to background levels (Figure 4, lane 7). Thus, FADD/MORT1 and caspase-8 are integral components of both the native homomeric TRAIL-R1 DISC and the native homomeric TRAIL-R2 DISC in BL60 cells.

FADD/MORT1 and Caspase-8 Are Essential for TRAIL-R2-Induced Apoptosis in Jurkat Cells

The mere presence of a protein in the DISC does not imply functional importance at the initiation of apoptosis. Other proteins have been shown to associate with CD95 or TNF-R1 (Krammer, 1999; Wallach et al., 1999). However, for the CD95 system, an essential function could so far only be attributed to FADD/MORT1 and

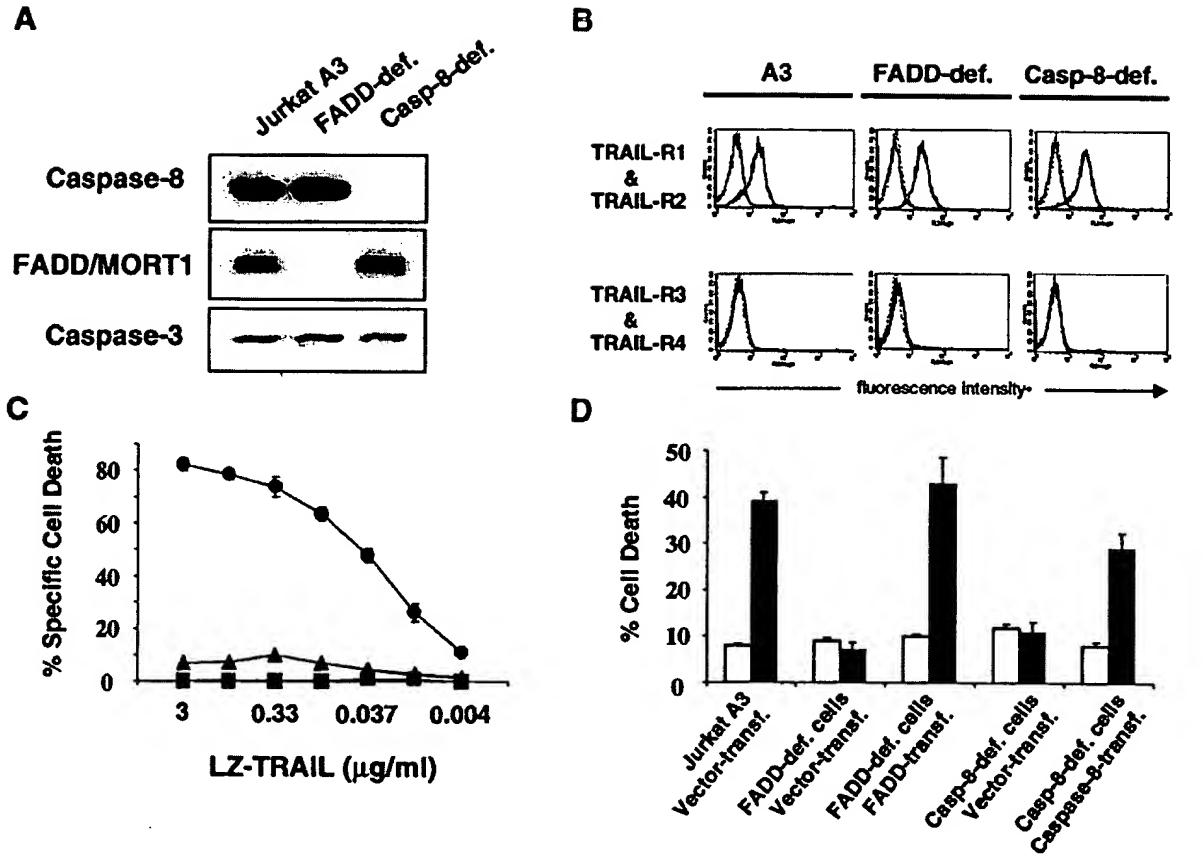

Figure 5. FADD/MORT1 and Caspase-8 Are Necessary for TRAIL-R2-Induced Apoptosis

(A) Western Blot analysis for expression levels of FADD/MORT1, caspase-8, and caspase-3 in Jurkat A3, FADD/MORT1^{def}, and caspase-8^{def} Jurkat cells. Migration positions of the detected proteins are indicated.

(B) FACS analysis of surface expression of TRAIL-R1 (upper panels, solid lines); TRAIL-R2 (upper panels, solid bold lines); TRAIL-R3 (lower panels, solid lines); and TRAIL-R4 (lower panels, solid bold lines) as compared to an isotype-matched control mIgG1 mAb (dashed lines) on Jurkat A3, FADD^{def}, and casp-8^{def} cells.

(C) Jurkat A3 (closed circle), FADD^{def} (closed square), and casp-8^{def} (closed triangle) cells were treated with the indicated concentrations of LZ-TRAIL or were left untreated. Cell death was determined 12 hr after stimulation. Percentage of specific cell death was calculated as follows: 100 × (% experimental cell death − % spontaneous cell death)/(100 − % spontaneous cell death). Spontaneous cell death was below 10% in all samples. All data points are the mean (± SD) of six independent experiments.

(D) Jurkat A3, FADD^{def}, and casp-8^{def} cells were transfected with an expression plasmid coding for a spectrin–GFP fusion protein (Kalejta et al., 1997) together with an expression plasmid coding for the respective missing signaling component or together with empty vector in the controls. Cells were incubated for 10 hr in the absence (open bars) or presence (closed bars) of 1 μg/ml LZ-TRAIL before cell death was determined by FSC/SSC analysis in the GFP-positive cell population. Mean ± SD of two independent experiments is shown.

caspase-8 as cells deficient for one of these two signaling proteins were resistant to CD95-mediated apoptosis. In addition to experiments with cells from mice deficient for FADD/MORT1 (Yeh et al., 1998; Zhang et al., 1998) or caspase-8 (Varfolomeev et al., 1998) that showed the requirement of these two signaling proteins, respectively, it was shown that Jurkat cells deficient for either one of these two proteins did not undergo CD95-mediated apoptosis (Juo et al., 1998, 1999). These FADD/MORT1-deficient (FADD^{def}) and caspase-8-deficient (casp-8^{def}) Jurkat cells expressed normal levels of caspase-3 but showed the expected deficiencies in FADD/MORT1 and caspase-8 expression, respectively (Figure 5A). In addition, the TRAIL receptor surface expression pattern of the deficient clones was identical to that of Jurkat A3 control cells as TRAIL-R2 was expressed while TRAIL-R1, -R3, and -R4 were not present (Figure 5B). In order to determine whether caspase-8 and FADD/MORT1 are necessary for TRAIL-R2-induced apoptosis in Jurkat cells, we treated FADD^{def}, casp-8^{def}, and Jurkat A3 control cells with LZ-TRAIL. Both the FADD^{def} and casp-8^{def} Jurkat cells were resistant to TRAIL-induced apoptosis, while Jurkat A3 cells underwent TRAIL-induced apoptosis in a dose-dependent manner (Figure 5C).

FADD/MORT1 and caspase-8 deficiency do not necessarily constitute the only defects of these mutated cell lines. Therefore, we tested whether ectopic expression of FADD/MORT1 or caspase-8 resensitized the respective Jurkat clones for TRAIL-induced apoptosis. While Jurkat A3 cells transfected with vector control were killed by LZ-TRAIL, both Jurkat FADD^{def} and Jurkat casp-8^{def} remained TRAIL resistant when transfected with vector alone. However, reconstitution of the miss-

ing protein resensitized both cell lines for TRAIL-induced apoptosis (Figure 5D). Since TRAIL resistance was overcome by reexpression of FADD/MORT1 in Jurkat-FADD^{def} and by reexpression of caspase-8 in the caspase-8^{def} Jurkat cells, TRAIL resistance was due to deficiency in the respective signaling proteins. Therefore, we conclude that FADD/MORT1 and caspase-8 are essential for TRAIL-R2-induced apoptosis and cannot be substituted for by other endogenous proteins in Jurkat cells.

Discussion

Thus far, it has been controversial whether the adaptor protein FADD/MORT1 and caspase-8 play a key role in TRAIL-induced apoptosis (Ashkenazi and Dixit, 1999). Our data demonstrate that endogenous FADD/MORT1 and caspase-8 are recruited to the native TRAIL DISC within seconds following stimulation with TRAIL. Subsequently, caspase-8 is cleaved and DNA fragmentation typical for apoptosis is observed in BL60, BJAB, CEM, and Jurkat cells. These data imply that FADD/MORT1 and caspase-8 are likely to play an important role during TRAIL-induced apoptosis.

However, FADD/MORT1-deficient murine embryonic fibroblasts (MEF) were shown to undergo apoptosis upon overexpression of human TRAIL-R1 (Yeh et al., 1998). In the light of our data showing essential recruitment of FADD/MORT1 and caspase-8 to the TRAIL-R2 DISC, there are three possible explanations for this finding by Yeh and colleagues. TRAIL-R1-induced apoptosis in MEFs could be due to overexpression-related unspecific recruitment of an adaptor protein different from FADD/MORT1. Alternatively, cell type–specific differences between the lymphoid cell lines studied here and MEFs could be responsible for the differential results. The most attractive explanation, however, is that TRAIL-R1 and TRAIL-R2 may be differentially regulated. TRAIL-R1-induced apoptosis may, therefore, use an additional, as of yet unidentified, FADD/MORT1-independent apoptotic mechanism (Yeh et al., 1998) apart from the FADD/MORT1 involving pathway (Figure 4), whereas TRAIL-R2-induced apoptosis may be entirely dependent on FADD/MORT1 (Figures 3, 4, and 5). Thus, the observed coexpression of the two apoptosis-inducing TRAIL receptors on certain cell types would allow for fine-tuned regulation of TRAIL-induced apoptosis.

DN-caspase-10 and not DN-caspase-8 (Pan et al., 1997a) or both DN-caspase-10 and DN-caspase-8 (MacFarlane et al., 1997) were reported to associate with overexpressed TRAIL-R1 and TRAIL-R2 and to inhibit apoptosis induced by overexpression of these two receptors. In addition, caspase-10 was suggested to play a role in TRAIL-induced apoptosis of mature DC and peripheral activated T cells. Deletion of these cells was reported to be inhibited in ALPS II patients carrying mutated caspase-10, which supposedly caused TRAIL resistance of T cells and DCs in these patients (Wang et al., 1999). However, recently one of the caspase-10 variants was identified as a common polymorphism in the Danish population with an allele frequency of 6.8% (Gronbaek et al., 2000). These data cast doubt on the role of caspase-10 mutations as the sole causative factor for ALPS II. In the light of our data on native TRAIL DISC composition, the concept that caspase-10 and not caspase-8 initiates TRAIL-induced apoptosis may not be generally applicable.

Other adaptors like RIP and TRADD, although prominently present in the cell lysates, could not be detected in the BL60 TRAIL DISC (Figure 2). Although we cannot rule out that these proteins may be associated as minor components or in a more transient fashion than FADD/MORT1 and caspase-8, they are probably less important for the initiation of TRAIL-induced apoptosis. In the case of TNF-R1 signaling, RIP and TRADD association have been shown to activate the gene inductive JNK and NF-κB pathways (Wallach et al., 1999). As we have only investigated TRAIL-sensitive cells, other proteins including RIP and TRADD, reported to associate with TRAIL receptors upon overepression, may serve anti- rather than proapoptotic functions. Analysis of TRAIL-resistant cells will provide further insight into these mechanisms.

So far, it was unclear whether TRAIL-induced apoptosis may be differentially regulated by the triggering of the two different apoptosis-inducing TRAIL receptors. Here we show that TRAIL-induced cross-linking of either TRAIL-R1 or TRAIL-R2 leads to the recruitment of both FADD/MORT1 and caspase-8 to the individual receptor-specific DISCs. Apart from the homomeric receptor complexes, heteromeric complexes consisting of TRAIL-R1 and TRAIL-R2 also may exist. We cannot exclude that these complexes may signal apoptosis via an additional pathway different from the FADD/MORT1 and caspase-8 involving pathway. However, these complexes and their associated signaling proteins would be part of the TRAIL DISC in BL60 cells. Since we did not detect TRADD, RIP, and caspase-10 in the BL60 TRAIL DISC (Figure 2), it is unlikely that these proteins associate with heteromeric complexes and may, thus, be common mediators of TRAIL-induced apoptosis.

Finally, we could show that TRAIL-resistant FADD/MORT1- and caspase-8-deficient Jurkat cells expressing TRAIL-R2 regained TRAIL sensitivity upon reexpression of these two proteins. Thus, caspase-8 and FADD/MORT1 are essential for TRAIL-R2-induced apoptosis, as they are for CD95-mediated apoptosis (Juo et al., 1998, 1999; Varfolomeev et al., 1998; Yeh et al., 1998; Zhang et al., 1998). These data clearly establish the importance of FADD/MORT1 and caspase-8 in TRAIL-induced apoptosis. FADD/MORT1 and caspase-8 have previously been shown to be implicated in apoptosis induction via CD95, TNF-R1, and TRAMP (Ashkenazi and Dixit, 1999; Krammer, 1999). Thus, the recruitment of endogenous FADD/MORT1 and caspase-8 to the native TRAIL-R1 and TRAIL-R2 provides evidence that these two proteins play a central role in death receptor–mediated apoptosis.

There is an apparent discrepancy between our data on the composition of the native TRAIL DISC and other reports showing no association of FADD/MORT1 with TRAIL-R1 or TRAIL-R2. In addition, in some studies overexpression of FADD-DN failed to inhibit apoptosis induced by TRAIL or overexpressed TRAIL-R1 or TRAIL-R2. While our studies were performed in cell lines expressing native levels of the respective proteins, all previous experiments were performed under conditions where at least one of the putatively interacting proteins

was overexpressed. This overexpression of DD/DED containing proteins might result in nonspecific aggregation. In addition, the homeostasis of different interacting proteins and, thus, assembly of the signaling complexes could be disturbed.

As shown in Figure 2, caspase-8 and FADD/MORT1 coprecipitate only with stimulated TRAIL receptors. Several of the previous studies relied on coprecipitation of proteins with overexpressed receptors without prior ligand-induced cross-linking. Although overexpression of TRAIL receptors induces apoptosis, this does not necessarily mimick ligand-induced receptor oligomerization. In conclusion, it seems that overexpression studies alone are not sufficient to define molecular interactions in apoptosis signaling.

TRAIL-induced apoptosis seems to utilize a pathway similar to the one used by CD95 (Krammer, 1999; Peter et al., 1999). Yet, most normal cells are resistant to TRAIL, whereas CD95 agonists have been shown to kill various normal cells (Nagata, 1997; Krammer, 1999) and, in contrast to TRAIL (Ashkenazi et al., 1999; Walczak et al., 1999), are toxic upon systemic administration (Ogasawara et al., 1993; Walczak et al., 1999). Thus, in addition to the similarities between the CD95 and TRAIL receptor pathways, there must also be differences between them. Studying TRAIL signaling in resistant cells will be likely to give insight into the mechanisms that regulate sensitivity versus resistance to TRAIL-induced apoptosis. The identification of the native TRAIL apoptosis-inducing signaling complex presented here provides the basis for such analyses.

Experimental Procedures

Cell Lines

The human B cell lines BL60 and BJAB and the human T cell lines CEM and Jurkat were maintained in RPMI 1640 (GIBCO-BRL, Karlsruhe, Germany) containing 10% fetal calf serum (GIBCO-BRL). The mutated Jurkat FADD^{def}, Jurkat casp-8^{def}, and the Jurkat A3 control cells were cultured as described elsewhere (Juo et al., 1998, 1999).

Antibodies and Reagents

Monoclonal antibodies (mAb) against FADD/MORT1, TRADD, RIP, and caspase-3 were purchased from Transduction Laboratories (San Diego, CA). Anti-caspase 10 mAb (Zytomed, Berlin, Germany) was raised against the p17 subunit of caspase 10. The mAb anti-FLICE C15 recognizes the p18 subunit of caspase-8 (Scaffidi et al., 1997), whereas the anti-caspase-3 mAb recognizes the p17 subunit of caspase-3 but not the p12 subunit. Leucine zipper (LZ) -TRAIL is a stable trimer of TRAIL and induces apoptosis upon binding to TRAIL-sensitive cells (Walczak et al., 1997). The antibodies specific for the different TRAIL receptors were described elsewhere (Griffith et al., 1999a). We used anti-TRAIL-R1 M272 and TRAIL-R2 M413 for FACS staining and anti-TRAIL-R1 M271 and anti-TRAIL-R2 M413 for receptor blockade. Combinations of anti-TRAIL-R1 mAbs (M270 and M272) or anti-TRAIL-R2 mAbs (M414 and M415) were used for Western blot detection of TRAIL-R1 and TRAIL-R2, respectively. Horseradish peroxidase (HRPO) –conjugated goat anti-mouse IgG1, IgG2a, and IgG2b polyclonal antibodies (pAb) were obtained from Southern Biotechnology Associates (Birmingham, AL). HRPO-goat anti-rat IgG was from Jackson ImmunoResearch (Dianova, Hamburg, Germany). All other chemicals used were of analytical grade and purchased from Merck (Darmstadt, Germany) or Sigma Chemical Co. (St. Louis, MO).

FACS Analysis

Cells were incubated with mAbs of the same isotype (mIgG1) against the four surface-expressed TRAIL receptors (M272 anti-TRAIL-R1, M413 anti-TRAIL-R2, M430 anti-TRAIL-R3, M444 anti-TRAIL-R4) or control mIgG1 followed by biotinylated secondary goat anti-mouse antibodies (Southern Biotechnology Associates) and Streptavidin-PE (Pharmingen, Hamburg, Germany). Surface staining was determined on a FACScan cytometer (Becton Dickinson, Heidelberg, Germany). Specificity of the respective anti-TRAIL-R mAbs used here was determined by staining of TRAIL-R1 to TRAIL-R4 on CV1/EBNA cells transfected with expression vectors coding for the individual surface-bound TRAIL receptors (data not shown).

Quantitation of Apoptotic Cell Death

As a direct measurement of apoptotic cell death, DNA fragmentation was quantified essentially as described (Nicoletti et al., 1991). Briefly, 2.5×10^5 cells were incubated in 24-well plates (Costar, Cambridge, MA) with or without apoptotic stimuli in 0.5 ml medium at 37°C. Cells were collected by centrifugation at 600 × g for 10 min at 4°C, washed twice with PBS, and then resuspended in 100 μl lysis solution containing 0.1% (v/v) Triton X-100, 0.1% (w/v) sodium citrate, and 50 μg/ml propidium iodide (PI). Apoptosis was quantitatively determined by flow cytometry after incubation at 4°C in the dark for at least 24 hr as cells containing nuclei with subdiploid DNA content. Alternatively, apoptosis was determined by a drop in the forward to sideward scatter (FSC/SSC) profile of apoptotic in comparison to living cells.

Preparation of Cell Lysates

Cells were harvested by centrifugation at 300 × g for 10 min at 4°C and washed twice with PBS, and lysates were prepared by resuspending the resulting cell pellets in 100 μl lysis buffer per 1×10^7 cells (30 mM Tris-HCl [pH 7.5], 150 mM NaCl, 10% Glycerol, 1% Triton X-100) supplemented with Complete protease inhibitors (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions. After 30 min incubation on ice, the lysates were centrifuged once at 15,000 × g at 4°C to remove nuclei. In the case of lysate preparation for ligand affinity precipitations, an intermediate centrifugation step (600 × g for 15 min at 4°C) was added after lysis in order to remove cellular debris.

Western Blot Analysis

For Western blot analysis, the resulting postnuclear supernatants or ligand affinity precipitates were supplemented with 2-fold concentrated standard reducing sample buffer (2× RSB). Subsequently, lysate containing 20 μg of protein as determined by the BCA method (Pierce, Rockford, IL) or proteins eluted from beads after ligand affinity immunoprecipitation were separated on 4%–12% NuPage Bis-Tris gradient gels (Novex, San Diego, CA) in MOPS buffer according to the manufacturer's instructions. After protein transfer onto nitrocellulose membranes (Amersham Pharmacia Biotech, Freiburg, Germany) by electroblotting, membranes were blocked with 5% nonfat dry milk (NFDM) in PBS/Tween (PBS containing 0.05% Tween-20) for at least 2 hr, washed with PBS/Tween, and incubated in PBS/Tween containing 3% NFDM and primary antibodies against caspase-3, caspase-10, FADD/MORT1, TRADD, RIP, TRAIL-R1 (M270 and M272), TRAIL-R2 (M414 and M415) (all at 1 μg/ml), or caspase-8 (1:10-diluted C15 hybridoma supernatant [Scaffidi et al., 1997]). Specificity of the respective anti-TRAIL-R mAbs used here was determined by Western blot analysis of TRAIL-R1 to TRAIL-R4 in lysates prepared from CV1/EBNA cells transfected with expression vectors coding for the individual TRAIL receptors (data not shown). After six washes for 5 min each in PBS/Tween, the blots were incubated with HRPO-conjugated isotype-specific secondary antibody diluted 1:20,000 in PBS/Tween. After washing six times for 5 min with PBS/Tween, the blots were developed by enhanced chemoluminescence (ECL) following the manufacturer's protocol (Amersham Pharmacia Biotech). For stripping, blots were either incubated for 30 min in a buffer containing 62.5 mM Tris/HCl (pH 6.8), 2% SDS, and 100 mM β-mercaptoethanol at 60°C or in 50 mM glycine HCl (pH 1.9) for 20 min at room temperature when only secondary antibodies needed to be removed. Subsequently, blots were washed six times for 10 min in PBS/Tween and blocked again.

Ligand Affinity Precipitation

We performed ligand affinity precipitations by using biotinylated LZ-TRAIL (Bio-LZ-TRAIL) in combination with Streptavidin beads (Pierce). Bio-LZ-TRAIL was prepared by incubation of purified LZ-TRAIL at 1 mg/ml with Sulfo-NHS-LC-Biotin at 1 mg/ml (Pierce) for 1 hr on ice before the reaction was stopped by adding 1/10 volume of 1 M Tris-HCl at pH 7.5. Unincorporated biotin was removed from Bio-LZ-TRAIL preparations by buffer exchange into 150 mM NaCl, 30 mM HEPES (pH 7.5) on PD-10 columns (Amersham Pharmacia Biotech). Protein preparations were checked for purity and incorporation of biotin by SDS-PAGE. The biological activity of Bio-LZ-TRAIL was determined by its apoptosis-inducing capacity and found to be comparable to nonbiotinylated LZ-TRAIL.

For ligand affinity precipitation, 3×10^8 cells were used per sample. Cells were washed twice with 50 ml RPMI medium at 37°C and subsequently incubated for the indicated time periods at 37°C and a cell density of 1×10^6/ml in the presence of 1 μg/ml Bio-LZ-TRAIL or, for the unstimulated control, in the absence of Bio-LZ-TRAIL. In the case of differential TRAIL receptor DISC analysis, we preincubated the cells with 10 μg/ml TRAIL-R1 and/or TRAIL-R2-blocking mAbs for 15 min before stimulation with Bio-LZ-TRAIL. DISC formation was stopped by addition at least 15 volumes of ice-cold PBS. Cells were then washed twice with 50 ml ice-cold PBS before cell lysates were prepared by addition of 4.5 ml lysis buffer per 3×10^8 cells. The resulting protein complexes were precipitated from the lysates by coincubation with 20 μl Streptavidin Beads (Pierce) for 2–4 hr on an end-over-end shaker at 4°C. For the precipitation of the nonstimulated receptors, Bio-LZ-TRAIL was added to the lysates prepared from nonstimulated cells at 1 μg/ml to control for protein association to nonstimulated receptor(s). Ligand affinity precipitates were washed four times with lysis buffer before the protein complexes were eluted from the beads by addition of 15 μl 2× standard reducing sample buffer. Subsequently, proteins were separated on SDS-PAGE before presence or absence of antigens was determined in the different precipitates by Western blot analysis.

Transfection and Complementation

Jurkat A3 control, FADD^{def}, and FLICE^{def} cells were transfected essentially as described (Juo et al., 1998) with slight modifications. In brief, 6×10^6 cells were transfected with a total of 20 μg DNA by electroporation with an expression plasmid coding for a spectrin–GFP fusion protein (Kalejta et al., 1997) together with an expression plasmid coding for the respective missing signaling component, or together with empty vector in the controls, at a ratio of 3 to 1 (0.4 cm cuvette, 250 V, 950 μF, Bio-Rad Gene-Pulser). The expression plasmids pRSV-HA-FADD, pRSV-caspase-8, or empty control vector are described elsewhere (Juo et al., 1998, 1999). Cells were purified over a Ficoll gradient 1 hr after transfection in order to remove dead cells. After each transfection, half of the cells were either left untreated or stimulated with 1 μg/ml LZ-TRAIL. After 10 hr of incubation, apoptosis was quantified by FSC/SSC analysis of the GFP-positive population on a FACScan cytometer (Beckton Dickinson).

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TRAIL induces death of human oligodendrocytes isolated from adult brain

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Summary

Tumour necrosis factor-related apoptosis-inducing ligand (TRAIL) has been reported to induce apoptosis in various tumour cell lines and, recently, also in normal cells. TRAIL interacts with four receptors: two signalling receptors (TRAIL-R1 and TRAIL-R2) and two decoy receptors (TRAIL-R3 and TRAIL-R4). We have shown that both signalling receptors are present on the surface of oligodendrocytes isolated from adult human brain (ahOL), whereas the decoy receptors are expressed at a low level on ahOL. TRAIL induces ahOL apoptosis—as characterized by Annexin V staining prior to propidium iodide cell uptake—under conditions of protein synthesis inhibition. However, pretreatment of ahOL with interferon γ (IFNγ) evoked susceptibility to TRAIL-induced death, which did not require inhibition of protein synthesis. A blocking experiment with monoclonal antibodies directed against TRAIL-R1 and TRAIL-R2 revealed that TRAIL-R1 is mainly involved in TRAIL-induced apoptosis of ahOL. In contrast to ahOL, microglial cells were completely resistant to cell death induced by TRAIL. Microglial cells had high surface expression of the decoy receptor TRAIL-R3, suggesting that resistance of these glial cells to TRAIL-induced death depends on the presence of the protective effect of TRAIL-R3. Stimulation of microglia with TRAIL increased further expression of TRAIL-R3, but it had no effect on the expression of TRAIL receptors by ahOL. This result may implicate TRAIL as an effector-immune molecule in selective ahOL demise in inflammatory/demyelinating conditions.

Keywords: TRAIL; oligodendrocytes; glial cells; death receptor; brain

Abbreviations: ahOL = adult human oligodendrocytes; CHX = cycloheximide; FITC = fluoroisothiocyanate; IFN = interferon; PI = propidium iodide; TNF = tumour necrosis factor; TRAIL = tumour necrosis factor-related apoptosis-inducing ligand; TRAIL-LZ = TRAIL leucine-zipper; TRAIL-R = TRAIL receptor

Introduction

Tumour necrosis factor-related apoptosis-inducing ligand (TRAIL) and its receptors form a potent ligand–receptor system, which is responsible for cell death. TRAIL is homologous to other death-signalling molecule family members such as Fas-L, tumour necrosis factor (TNF) and lymphotoxin (LT)-α and LTβ (Pitti *et al.*, 1996). The unique feature originally attributed to TRAIL was selective TRAIL-induced apoptosis of tumourigenic or transformed cells, but not normal cells (Willey *et al.*, 1995). More recently, however, it has been shown that TRAIL can induce apoptosis of normal hepatocytes, indicating that TRAIL-induced cell death is not restricted to transformed cells (Jo *et al.*, 2000). These results widened the potential pathogenic role of TRAIL to other conditions involving cell death such as inflammation and autoimmunity. TRAIL is expressed widely in many cell types and tissues, and the regulation of TRAIL-induced death is through restricted expression of its receptors (Pan *et al.*, 1997). Four TRAIL receptors (TRAIL-Rs) have been cloned: TRAIL-R1 and TRAIL-R2 are functionally active receptors, whereas TRAIL-R3 and TRAIL-R4 are non-signalling decoy receptors. The mutual expression of signalling and decoy receptors determines whether cells are sensitive or resistant to TRAIL-induced death (Degli-Esposti *et al.*, 1997; Sheridan *et al.*, 1997; Walczak *et al.*, 1997). However, the hypothesis is not entirely accurate because there is no obvious correlation between the mRNA for TRAIL-R3 and TRAIL-R4 and resistance to TRAIL-induced death in many tumour cell lines (Griffith *et al.*, 1998).

Little is known about TRAIL-induced effects in the CNS. Several CNS-related conditions involve neuronal and glial cell death. Recently, Nitsch and colleagues (Nitsch *et al.*, 2000) used a brain slice culture system to show that TRAIL

induced extensive and non-selective brain cell death. These results may indicate particular sensitivity of CNS cells to TRAIL. Depletion of oligodendrocytes is a recognized feature of multiple sclerosis lesions. Several immune mechanisms have been proposed to induce oligodendrocyte death including non-major histocompatibility complex (MHC) restricted injury by other TNF family members such as Fas-L, TNF and LTα (Raine, 1994; Brosnan and Raine, 1996; Selmaj and Raine, 1998). These results indicate that death receptor ligation may provide a major destructive signal to oligodendrocytes in autoimmune inflammatory lesions.

In this study, we have assessed (i) the expression of TRAIL-Rs on oligodendrocytes isolated from adult human brain (ahOL) and microglial cells; and (ii) the effect of TRAIL on ahOL and microglial cell survival.

Material and methods

Reagents

TRAIL leucine-zipper (TRAIL-LZ) and mouse monoclonal antibodies against human TRAIL(M180), TRAIL-R1(M271), TRAIL-R2(M413), TRAIL-R3(M430), TRAIL-R4(M444) were a generous gift from Immunex Corporation (Seattle, WA, USA). The antibodies' specificity for different TRAIL-Rs has been described previously (Griffith *et al.*, 1999). Briefly, antibodies against TRAIL-Rs were generated after immunization of mice with fusion proteins that consisted of the extracellular fraction of TRAIL-Rs linked to a constant region of human IgG1. Specificity of these antibodies was confirmed by flow cytometry and western blot analysis performed on CV-1 cells transfected with cDNA encoding the whole sequence of each receptor. No cross-reactivity could be detected in these assays.

Anti-mouse horseradish peroxidase (HRP) antibody (Santa Cruz, CA, USA), anti-mouse fluoroisothiocyanate (FITC) conjugate antibody and propidium iodide (PI) were obtained from Sigma (Poznan, Poland). Annexin V–FITC was purchased from Pharmingen (San Diego, CA, USA), interferon γ (IFNγ) from R&D Systems (Minneapolis, MN, USA), TNF from Endogen (Boston, CT, USA) and the human cDNA library from Clontech (Franklin Lakes, NJ, USA).

Target cells

Adult human oligodendrocytes (ahOL) obtained from neurosurgical procedures were prepared from adult human brain resected as a surgical treatment for tumours as described previously (Jurewicz *et al.*, 1998). Briefly, the tissue was treated with trypsin, rubbed through a mesh and centrifuged on a 30% Percoll gradient. The dissociated cells were suspended in minimum essential medium with 5% foetal calf serum (FCS), streptomycin (50 μg/ml) and penicillin (50 U/ml) (all from Gibco BRL, Life Technologies, Paisley, UK) before being cultured for 48 h in a culture flask. This step enabled adherent cells such as microglia and astrocytes to be separated from non-adherent cells such as oligodendrocytes. The non-adherent oligodendrocyte fraction was plated at a concentration of 5×10^4 cells/well onto 96-well microtitre plates coated with poly-L-lysine and cultured for 2 weeks. The purity of these cultures was ~90% (Jurewicz *et al.*, 1998). After purification, the cells (ahOL and microglia) were kept in culture for the same period of time before use in the experiments.

A cell line (MO3.13) formed by the fusion of ahOL and a rhabdomyosarcoma cell line (a generous gift from Dr Neil Cashman) was cultured in high glucose Dulbecco-modified Eagle's medium supplemented with 10% FCS, 2.5 U/ml penicillin, 2.5 μg/ml streptomycin and 2 mM L-glutamine at 37°C. A T98G glioma cell line was cultured under the same conditions. HELA and Jurkat cell lines were cultured in RPMI (Roswell Park Memorial Institute medium) with 10% FCS, 2.5 U/ml penicillin and 2.5 μg/ml streptomycin.

Before the experiments, all cell lines were seeded on 24-well plates, left for 24–48 h and then stimulated with TRAIL. ahOL were stimulated with TRAIL in a serum-deprived condition, 24 h pre-treatment with cycloheximide (CHX) (10 μg/ml) or IFNγ (100 U/ml). All cells were stimulated with 300–2000 ng/ml TRAIL-LZ (Immunex).

In the blocking experiment, ahOL pre-treated with CHX were incubated with anti-TRAIL-R1 or/and anti-TRAIL-R2 monoclonal antibodies (10 μg/ml) for 1 h before exposure to TRAIL.

Annexin V–FITC conjugation and PI cell staining

Annexin V–FITC and PI were used to determine the percentage of cells undergoing apoptosis after exposure to TRAIL. After incubation with TRAIL for 5, 24, 48 and 72 h, the cells were washed twice with cold phosphate buffered saline (PBS) pH 7.4 and then resuspended in calcium-containing binding buffer (Pharmingen) at a concentration of 1×10^6 cells/ml. Next, 5 μl of FITC-conjugated Annexin V and PI (5 μg/ml) were added in a calcium-containing buffer (Pharmingen) to 100 μl of cell suspension. After 15 min incubation in the dark at room temperature, the cells were immediately analysed by flow cytometry (see below). As described by Vermes and colleages (Vermes *et al.*, 1995), the apoptotic cells were defined as showing positive Annexin V staining prior to the appearance of PI staining.

Determination of surface expression by flow cytometry

Surface expression of TRAIL-Rs was determined by flow cytometric analysis by measuring the binding of anti-TRAIL-Rs monoclonal antibodies (M271, M413, M430, M444) (Immunex). Cells were incubated with TRAIL (300 ng/ml), TNF (1000 U/ml) and IFNγ (100 U/ml) for 24 h prior to analysing the expression of the receptors. Briefly, cells were

incubated with monoclonal antibodies for TRAIL-Rs (3 μg/ml) for 1 h on ice, and then with anti-mouse FITC-conjugated antibody (Sigma) (1 : 100) for 30 min on ice (dilution with PBS). After several washes with PBS, cells were analysed using a fluorescence-activated cell sorter (FACS) (Becton Dickinson, San Jose, CA, USA).

Western blotting

Cells were lysed in TBS buffer (Tris buffered saline: 0.05 M Tris, 0.138 M NaCl, 0.0027 M KCl, pH 8.0) containing phenylmethylsulphonyl fluoride, aprotinin and Triton X-100 at 25°C. The lysates were centrifuged at 14 000 *g* to remove cellular debris. Proteins were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), transferred to a polyvinylidene fluoride membrane (Millipore Corp., Bedford, MA, USA) and blocked with 5% non-fat dry milk (NFDM) in PBS–Tween-20 (0.05%) overnight at 4°C. The membrane was immunoblotted with monoclonal antibody directed against TRAIL-Rs (1 μg/ml in 5% NFDM in PBS–Tween-20) for 1 h as described previously (Griffith *et al.*, 1999). After washing, the membrane was incubated for 1 h with an anti-mouse HRP antibody. Following several washes, the blots were developed by chemiluminescence with ECL Plus according to the manufacturer's protocol (Amersham Pharmacia, Little Chalfont, UK).

Reverse transcription–polymerase chain reaction (RT–PCR) for TRAIL-Rs

Total RNA was isolated from cells using an RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. cDNA synthesis was performed using an oligo(dT) primer. Reverse transcription was performed using a thermal programme of 25°C for 10 min, 42°C for 30 min and 95°C for 5 min. PCR was performed using the following primers: β-actin (forward: 5′-GAAACTACCTTC-

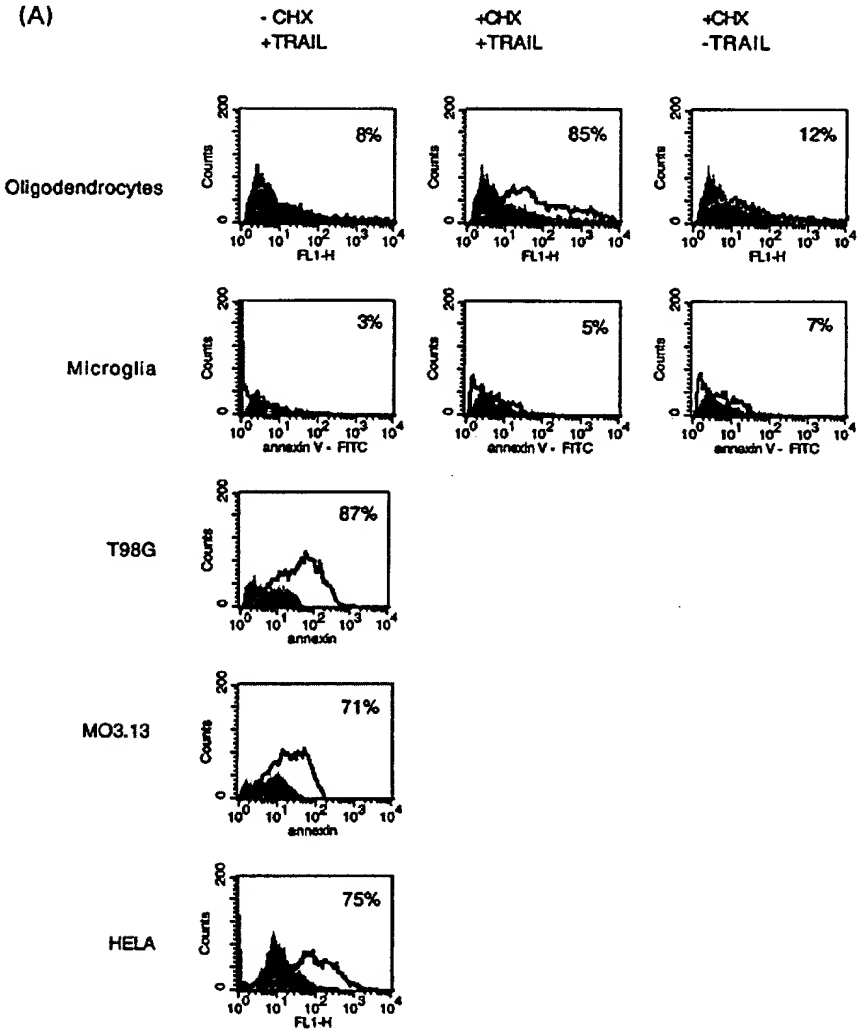


Fig. 1a

AACTTCCATC-3′, reverse: 5′-CGAGGCCAGGATGGAGCCGCC-3′); TRAIL-R1 (forward: 5′-CTGAGCAACGCAGACTCGCTGTCCAC-3′; reverse: 5′-TCCAAGGACACGGCAGAGCCTGTGCCAT-3′); TRAIL-R2 (forward: 5′-GCCTCATGGACAATGAGATAAAGGTGGT-3′, reverse: 5′-CCAAATCTCAAAGTACGCACAAACGG-3′); TRAIL-R3 (forward: 5′-GAAGAATTTGGTGCCAATGCCACTG-3′, reverse: 5′-CTCTTGGACTTGGCTGGGAGATGTG-3′); TRAIL-R4 (forward: 5′-CTTTTCCGGCGGCGTTCATGTCCTTC, reverse: 5′-GTTTCTTCCAGGCTGCTTCCCTTTGTAG); TRAIL (forward: 5′-CAACTCCGTCAGCTCGTTAGAAAG-3′, reverse: 5′-TTAGACCAACAACTATTTCTAGCACT-3′).

Human β-actin PCR cycle conditions were 95°C for 45 s, 55°C for 1 min, and 72°C for 45 s for 30 cycles. Human TR-1, TR-2 and TR-3 conditions were 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min for 30 cycles. Human TR-4 cycle conditions were 95°C for 4 min 15 s, followed by 30 cycles of 95°C for 45 s, 60°C for 45 s, and 72°C for 45 s (Griffith *et al.*, 1998). Human TRAIL cycle conditions were 95°C for 45 s, 55°C for 45 s, and 72°C for 45 s for 30 cycles (Fanger *et al.*, 1999). Samples were resolved on a 1% agarose gel and visualized with ethidium bromide.

Results

TRAIL induces ahOL apoptotic cell death

To determine whether oligodendrocytes and microglial cells are sensitive to TRAIL-LZ-induced apoptosis, we used Annexin V and PI staining, and cell analysis by flow cytometry to detect externalization of phosphatidylserine and PI uptake (indicating membrane disruption). When exposed to TRAIL-LZ at a concentration of 300–2000 ng/ml for up to 72 h, ahOL were resistant to cell death (Fig. 1A and B). Serum deprivation condition, which is known to increase cell susceptibility to other death receptor ligands (D'Souza *et al.*, 1995), did not decrease the survival of ahOL

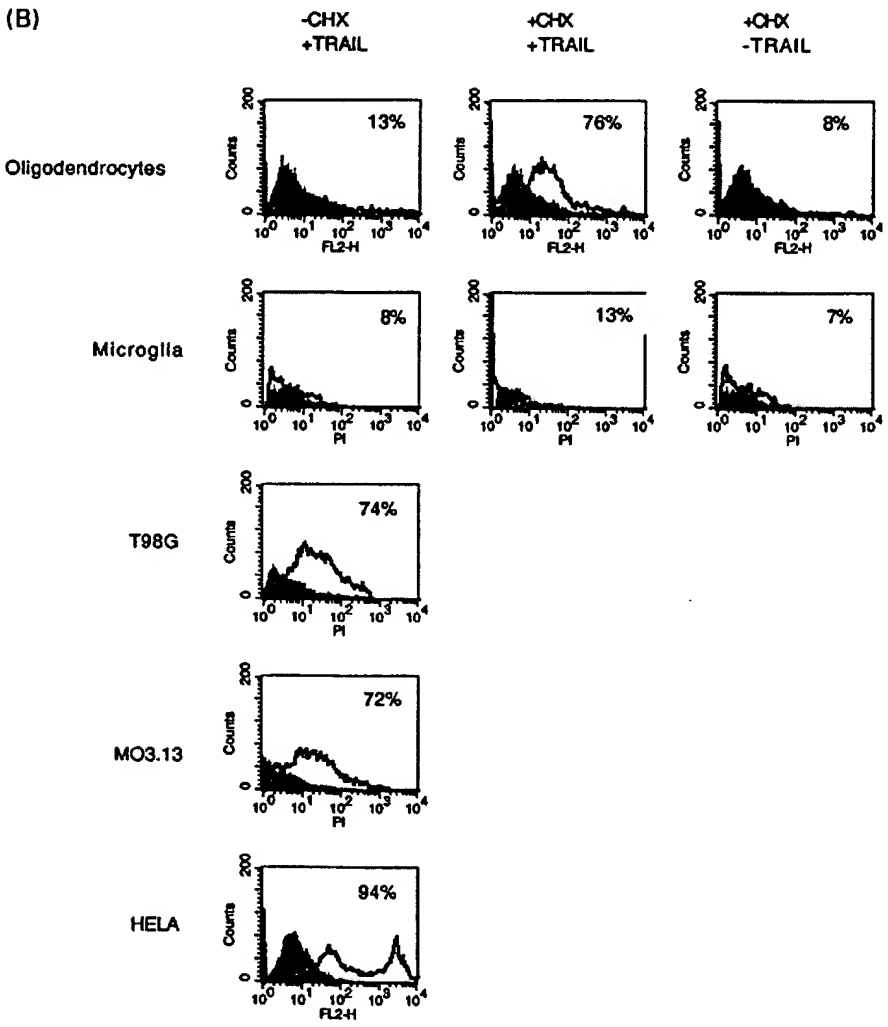


Fig. 1b

treated with TRAIL (data not shown). However, when ahOL were pre-treated with a protein synthesis inhibitor, CHX (10 μg/ml), for 24 h prior to TRAIL exposure, apoptosis was observed as detected by Annexin V staining preceding to PI cell uptake (Fig. 1A and B). Positive Annexin V–FITC staining of ahOL was observed 24 h after TRAIL exposure whereas PI uptake was detected only after 72 h. Cells that stained positively for Annexin V–FITC and negative for PI were considered to be undergoing apoptosis, whereas cells that stained positively for both Annexin V–FITC and PI were considered to be either in the end stage of apoptosis or already dead (Vermes *et al.*, 1995). We continued ahOL observation for up to 120 h and did not see cell recovery, indicating that TRAIL-induced cell death and increased permeability for PI was irreversible. Regardless of protein synthesis inhibition, microglial cells were completely resistant to TRAIL-LZ for up to 72 h of incubation (Fig. 1A and B). As a control, we used three human cell lines representing transformed cells MO3.13, T98G and HELA, respectively. All these cell lines died within 24 h after TRAIL stimulation (Fig. 1A and B) and their death did not require inhibition of protein synthesis. This agrees with published results indicating high susceptibility of transformed cells to TRAIL-induced death (Kim *et al.*, 2000). Since IFNγ was implicated in increasing cell death susceptibility to other TNF family ligands, we assessed whether pre-treatment of ahOL and microglia cells with IFNγ evoked cell

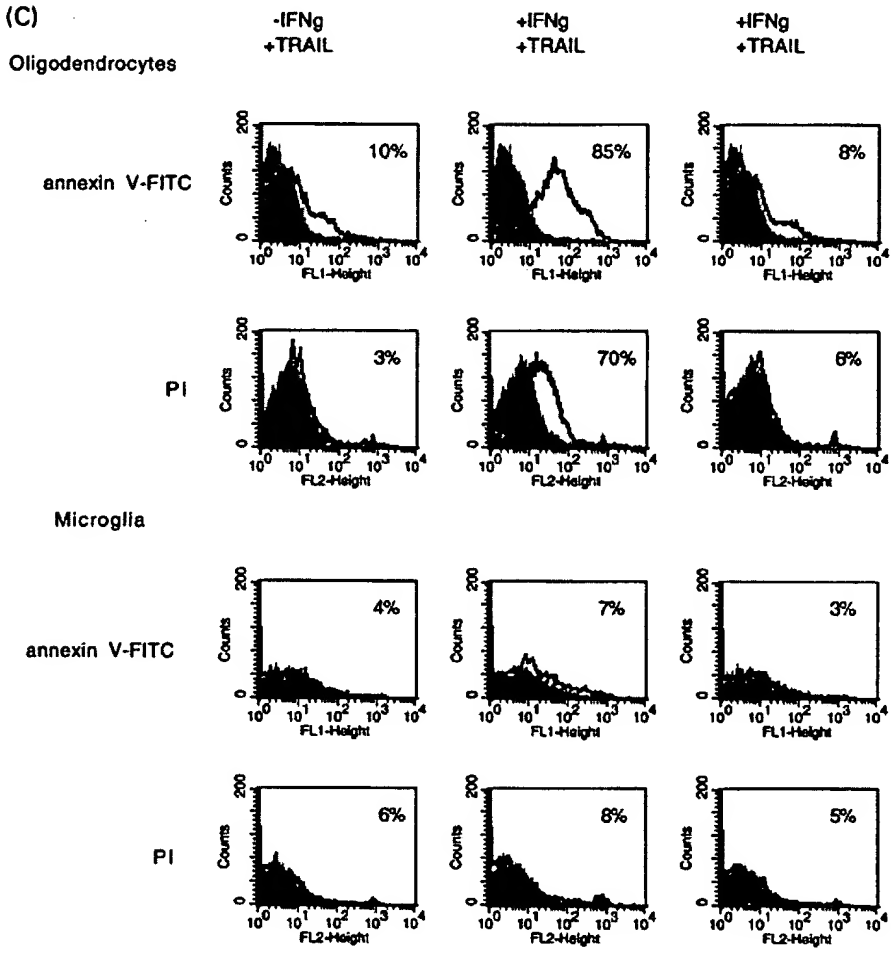
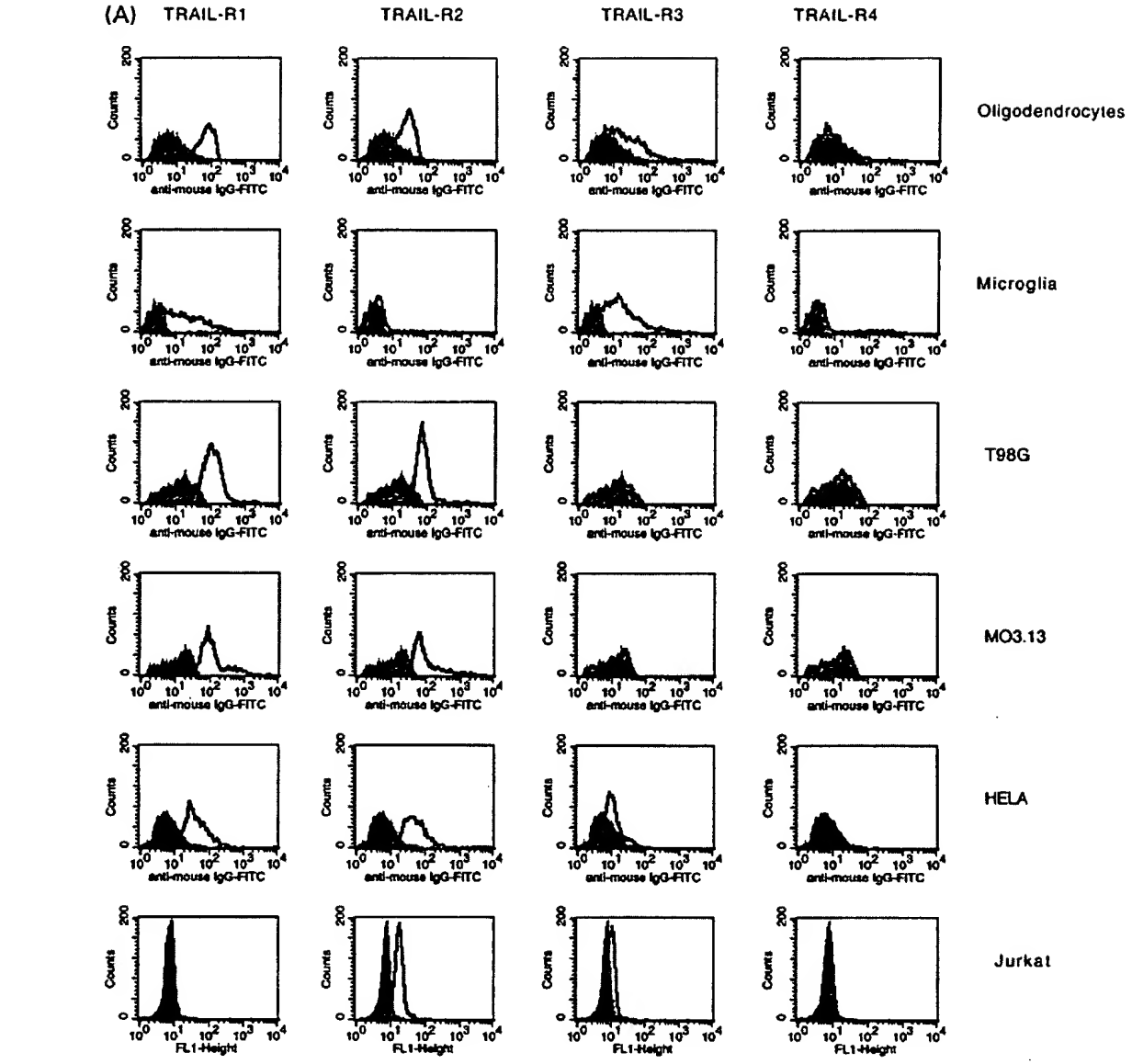


Fig. 1 TRAIL induces apoptosis of ahOL under conditions of protein synthesis inhibition or pre-treatment with IFNγ. (**A**) Annexin V–FITC staining 24 h after TRAIL (300 ng/ml) exposure of ahOL and microglia, and 5 h after TRAIL exposure of T98G, MO3.13 and HELA cell lines. (**B**) PI staining 72 h after TRAIL (300 ng/ml) exposure of ahOL and microglia, and 24 h after TRAIL exposure of T98G, MO3.13 and HELA cell lines. (**C**) Annexin V staining 24 h after TRAIL exposure and PI staining 72 h after TRAIL exposure of ahOL and microglia cells pre-treated with 100 U/ml of IFNγ for 24 h. Histograms represent fluorescence intensity on the horizontal axis and relative cell number (counts) on the vertical axis. All histograms represent stimulated cells (thick, black lines) (as indicated above the histogram) and non-stimulated cells (grey, shadow peaks). One of six representative experiments is shown. Percentages indicate the proportion of dead cells. This figure can be viewed in colour as supplementary material at Brain Online.



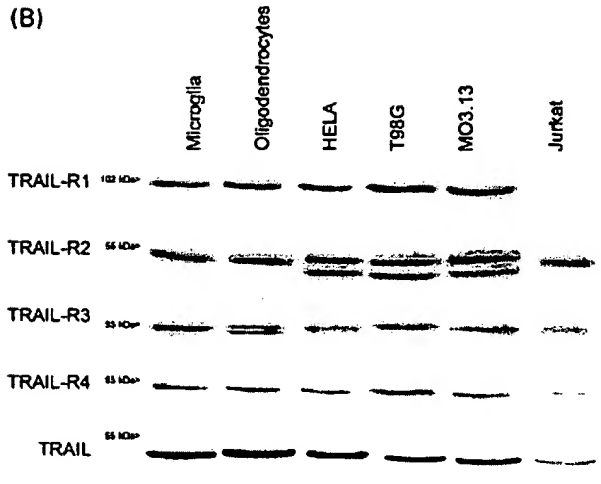


Fig. 2 TRAIL-R expression in glial cells. (A) FACS analysis of surface expression of TRAIL-Rs using specific mouse monoclonal antibodies (thick, black lines) compared with the isotype control (shadow peaks). Histograms represent fluorescence intensity on the horizontal axis and relative cell number (counts) on the vertical axis. (B) TRAIL-R and ligand expression in ahOL, microglial cells, MO3.13, T98G, HELA and Jurkat cell lines were determined by western blotting as described in Material and methods. One of five representative experiments is shown. Part A of this figure can be viewed in colour as supplementary material at Brain Online.

death induced with TRAIL. Figure 1C shows that IFNγ added 24 h prior to TRAIL made ahOL susceptible to TRAIL-induced cell death. The susceptibility to ahOL death induced by IFNγ in response to TRAIL may be related to decreased expression of TRAIL-R3 (see below).

Expression of TRAIL-Rs in glial cells

We used western blotting and flow cytometry to (i) determine the correlation between the expression of TRAIL-Rs in glial cells and their susceptibility to TRAIL-mediated cell death; and (ii) define their surface or intracellular localization. Using these two methods, we found that ahOL, microglia and the control lines MO.313, T98G and HELA all expressed TRAIL-R1 and TRAIL-R2 both on the cell surface and intracellularly (Fig. 2A and B). As previously reported (Sprick *et al.*, 2000), the Jurkat cell line expressed only TRAIL-R2 and a low level of TRAIL-R3 both on the cell surface and intracellulary. Interestingly, however, ahOL and microglial cells had a single band of immunoreactivity for TRAIL-R2 on the western blot compared with the double bands showed by most of the transformed control cell lines. This may indicate differences in the function of TRAIL-R2 between normal and transformed cells (see below). Microglial cells, which are resistant to TRAIL-induced death, expressed high levels of TRAIL-R3 on the cell surface, whereas ahOL expressed low levels of TRAIL-R3 and MO3.13, T98G and HELA cells did not express TRAIL-R3 (Fig. 2A). However, western blotting detected expression of TRAIL-R3 in all the studied cell populations (Fig. 2B). These results may indicate facilitated translocation of TRAIL-R3 from an intracellular compartment to the cell surface in microglia cells, which are resistant to TRAIL-induced death, but not in the other cells which are sensitive to TRAIL-induced death. The expression of TRAIL-R4 on the cell surface was absent from all the studied cell populations, but weak immunoreactivity was detected with western blotting (Fig. 2B).

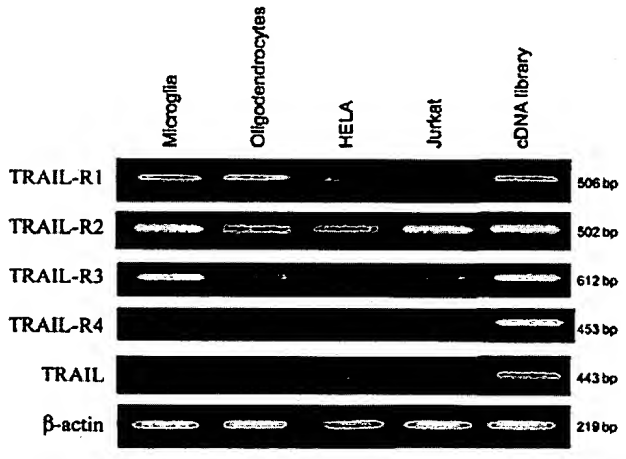


Fig. 3 RT–PCR analysis of mRNA expression of TRAIL and TRAIL-Rs in glial cells. Total RNA was extracted from ahOL, microglial cells, HELA cells and Jurkat cells. RT–PCR was performed using the specific oligoprimers listed in Material and methods. Samples were resolved on a 1% agarose gel and visualized with ethidium bromide. One of three representative experiments is shown.

mRNA expression of TRAIL-Rs in glial cells

To further investigate the function of TRAIL-Rs in glial cells, we measured their transcription efficacy by assessing mRNA expression for TRAIL-R1, TRAIL-R2, TRAIL-R3 and TRAIL-R4. Microglia cells, ahOL, HELA and Jurkat cells were strongly positive for TRAIL-R2 mRNA. In addition, expression of mRNA encoding TRAIL-R1 was detected in ahOL and microglial cells. TRAIL-R3 mRNA was expressed strongly in microglia; this correlated with its surface expression at a protein level in these cells (see above). Jurkat cells also expressed TRAIL-R3 mRNA. All investigated cells were negative for TRAIL-R4 mRNA, although the specificity of PCR reaction was confirmed with the human cDNA library (Fig. 3).

TRAIL-induced ahOL death is mediated by TRAIL-R1

To assess which receptor is involved in the TRAIL-induced death of ahOL, we performed an experiment in which we added blocking antibodies against TRAIL-R1 and/or TRAIL-R2 for 1 h prior to TRAIL exposure. Then we determined cell death by staining the cells with Annexin V and PI. These experiments showed that blocking with TRAIL-R1 antibody protects ahOL from cell death whereas the antibody to TRAIL-R2 had no effect on TRAIL-induced apoptosis of ahOL (Fig. 4). There was also no synergistic effect when both anti-TRAIL-Rs were added. We used Jurkat cells, which expressed only TRAIL-R2, as a positive control of antibody against TRAIL-R2 (Sprick *et al.*, 2000). We observed a strong inhibitory effect of this antibody on the TRAIL-induced death of Jurkat cells (Fig. 4). These results indicate that the death signal in ahOL depends on the TRAIL–TRAIL-R1 interaction.

Regulation of the expression of TRAIL-Rs in glial cells

Previous results have shown that cytokines can down-regulate or up-regulate TRAIL and TRAIL-R expression (Kayagaki *et al.*, 1999*a*, *b*; Sedger *et al.*, 1999). To assess regulation of TRAIL-R expression in glial cells, we measured TRAIL-Rs on the surface of ahOL and microglia after stimulation with TNF and IFNγ; no increase in the expression of TRAIL-Rs was observed (data not shown). When TRAIL ligand was added, however, we observed strong up-regulation

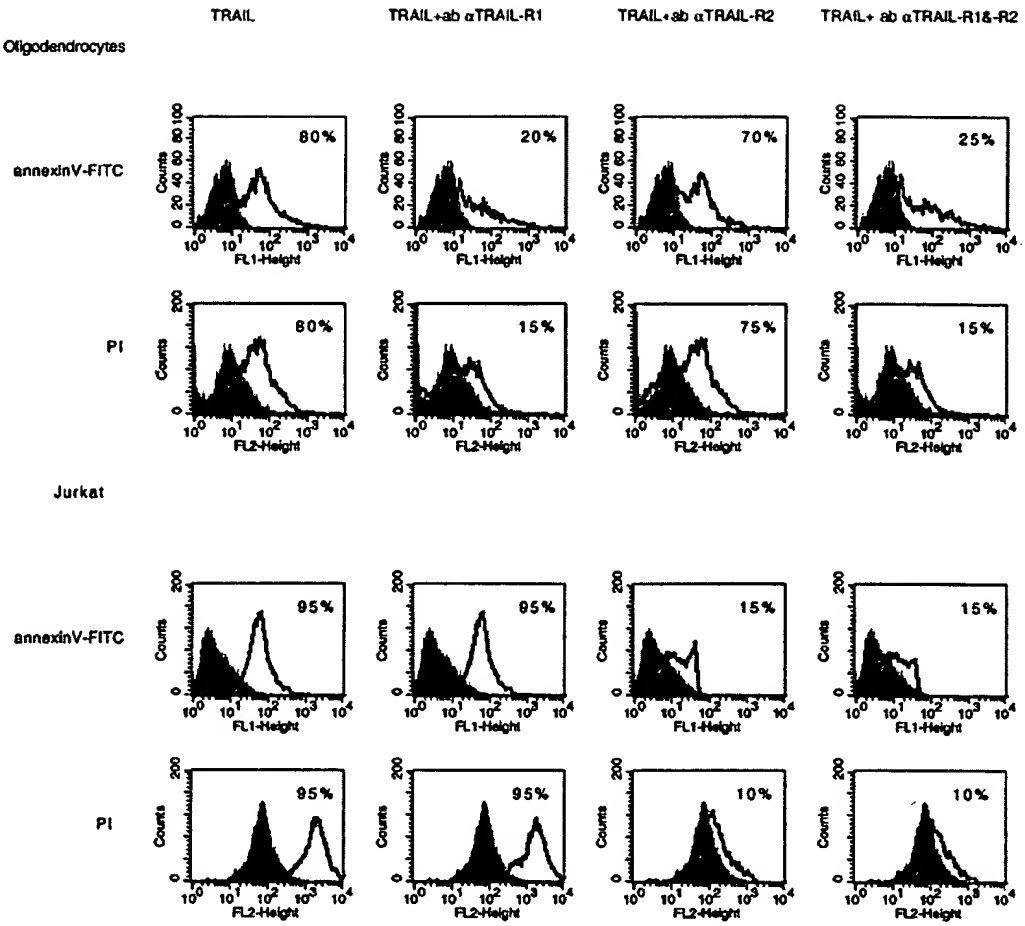


Fig. 4 TRAIL-induced ahOL death is mediated by TRAIL-R1. ahOL and Jurkat cells were incubated with TRAIL-R1(M271) antibody and/or TRAIL-R2(M413) antibody for 1 h prior to exposure to TRAIL-LZ (300 ng/ml). ahOL were pre-incubated with CHX for 24 h prior to TRAIL exposure, but Jurkat cells were not pre-treated. All histograms represent stimulated cells (thick, black lines) (as indicated above the histogram) and non-stimulated cells (grey, shadow peaks). Histograms represent fluorescence intensity on the horizontal axis and relative cell number (counts) on the vertical axis. One of three representative experiments is shown. Percentages indicate the proportion of dead cells. This figure can be viewed in colour as supplementary material at Brain Online.

of TRAIL-R3 expression on microglia cells but not on ahOL cells (Fig. 5). These results indicate that TRAIL ligand can up-regulate TRAIL-R3 expression on microglia in an autoregulatory manner and protect the cells from death. Interestingly, adding IFNγ to ahOL decreased the already low expression of TRAIL-R3; these results correlated with the IFNγ-induced susceptibility to TRAIL-mediated death of ahOL (Fig. 5).

Discussion

In this study, we have shown that both the TRAIL cell death mediating receptors, TRAIL-R1 and TRAIL-R2, are expressed by ahOL isolated from human adult brain and that ligation of TRAIL-R1 induces ahOL death in the presence of protein synthesis inhibition or pre-treatment with IFNγ. The susceptibility to TRAIL-induced death demonstrated by ahOL depends on low expression of decoy TRAIL-R3. This was in contrast to microglia cells, which expressed high levels of TRAIL-R3 and were resistant to TRAIL-induced death.

In other cell culture systems, TRAIL-induced death has also been shown to be evoked or enhanced by protein synthesis inhibitors such as CHX (Bretz *et al.*, 1999) or actinomycin D (Griffith *et al.*, 1998). This suggests that CHX and actinomycin D reduce the relative concentration of labile protein inhibitors in the TRAIL transduction pathway. The family of IAP (inhibitor of apoptosis protein) molecules has been shown to protect cell death induced by TNF family ligands; these molecules bind to and inhibit the activity of caspases—preferentially caspase-3 and caspase-9 (Ducket *et al.*, 1996). Similarly, FLIP [FADD-like ICE (FLICE) inhibitory protein] inhibits the death receptor inducing signal by preventing caspase-8 and caspase-10 association with the adapter molecule FADD (Inohara *et al.*, 1997; Irmler *et al.*, 1997). It has recently been shown that ligation of TRAIL-R1

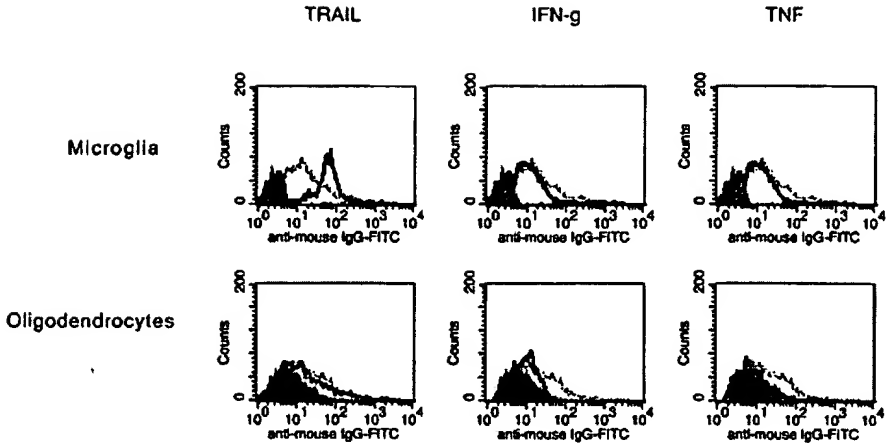


Fig. 5 Regulation of TRAIL-R3 expression in glial cells. Microglia and ahOL were stimulated with TRAIL (300 ng/ml), IFNγ (100 U/ml) and TNF (1000 U/ml) for 24 h as described in Material and methods. TRAIL induces enhanced expression of TRAIL-R3 on microglial cells, but not on ahOL (thick, black line). TNF did not influence TRAIL-R3 expression, but IFNγ decreased TRAIL-R3 expression on ahOL (thick, black line). FACS analysis of surface expression of TRAIL-R3 is shown for non-stimulated cells (dotted, black line) compared with TRAIL-R3 expression on stimulated cells (TRAIL, TNF, IFNγ; thick, black line) and the isotype control (grey, shadow peaks). Histograms represent fluorescence intensity on the horizontal axis and relative cell number (counts) on the vertical axis. One of the four representative experiments is shown. This figure can be viewed in colour as supplementary material at Brain Online.

and TRAIL-R2 initiated FADD and caspase-8 association (Kischkel *et al.*, 2000) and, accordingly, FLIP has been reported to inhibit TRAIL signalling (Wajant *et al.*, 2000). In addition, the sensitivity of tumour cells to TRAIL was linked with the fact that they express low levels of FLIP (Kim *et al.*, 2000). We have also detected expression of FLIP in ahOL and found that CHX decreased endogenous FLIP protein levels rapidly (data not shown). All these data indicate that TRAIL, despite being primarily toxic to transformed cells, can also mediate normal cell death—including that of ahOL—under appropriate conditions (mainly related to the inhibition of protein synthesis). Of particular importance is that susceptibility to TRAIL-induced death of ahOL can also be evoked by pre-treatment with IFNγ. The synergistic activity of IFNγ with TNF family ligands has been shown previously and was suggested to be related to the IFNγ-induced expression of TNF receptors (Pouly *et al.*, 2000). In this study, however, we have shown that IFNγ-induced susceptibility of ahOL to TRAIL depends on the decreased expression of a decoy TRAIL-R3.

The observation that TRAIL can induce selective cell death of ahOL may be of importance to inflammatory/demyelinating conditions (including multiple sclerosis) in which the demise of oligodendrocytes is a recognized pathological feature. Thus, TRAIL can be added to the family of immune effector molecules that can be instrumental in ahOL demise. TRAIL, like TNFα and FasL, has both membrane and trimeric soluble forms (Pitti *et al.*, 1996). Although TRAIL mRNA has been found in various tissues and cells, both its expression at protein levels and its physiological function are still largely unknown. TRAIL expression by activated T lymphocytes and natural killer (NK) cells suggests that it may be involved in cell-mediated cytotoxicity (Kashii *et al.*, 1999; Kayagaki *et al.*, 1999*c*). TRAIL production has been shown to be under control of cytokines, e.g. type I interferons induced TRAIL expression on human T cells; IFNγ up-regulated TRAIL expression on murine liver NK cells; and interleukin-2 (IL-2) and IL-15 induced TRAIL expression on murine spleen NK cells (Kayagaki *et al.*, 1999*a*, *b*; Sedger *et al.*, 1999). Lipopolysaccharide increased TRAIL expression on human monocytes (Halaas *et al.*, 2000). In addition, TRAIL was detected on autoimmune T cells (Wendling *et al.*, 2000), which can use it as an effector molecule against CNS targets. Unlike soluble TNF receptors (Selmaj and Raine, 1995), however, a soluble TRAIL receptor inducing TRAIL blockade exacerbated experimental autoimmune encephalomyelitis (Hilliard *et al.*, 2001). Interestingly, the CNS of mice treated with a soluble TRAIL receptor did not contain any more apoptotic cells, but lymphocyte production of cytokines in response to the encephalitogenic antigen, myelin oligodendrocyte glycoprotein (MOG), was increased (including both Th1 and Th2 types of cytokine). It was concluded that TRAIL might act differently to other TNF family ligands in experimental autoimmune encephalomyelitis and inhibit this disease. However, an alternative explanation may be that TRAIL inhibition prolonged survival of lymphocytes in the peripheral lymphatic organs leading to their prolonged activation and enhancement of experimental autoimmune encephalomyelitis. It is not clear whether the soluble TRAIL receptor construct was able to pass through the blood brain

barrier, enter the brain and interfere with TRAIL's interaction with oligodendrocytes.

Our results have clearly shown that TRAIL induced selective death of oligodendrocytes; this was underscored by the inability to kill microglia cells, a companion glial cell population. Recently, Nitsch and colleagues (Nitsch *et al.*, 2000) reported that incubation of human brain slices with human recombinant TRAIL trimerized with a FLAG-specific antibody resulted in extensive brain cell death (as measured by nuclei staining with PI). PI-labelled cells in TRAIL-treated brain slices were identified by double-fluorescence immunocytochemistry as neurones, oligodendrocytes, astrocytes and microglial cells. These results indicated that TRAIL evoked a general destructive effect against neurones and glial cells in this brain slice system.

Extensive studies of the sensitivity of CNS cells to other members of the TNF family have shown great selectivity in their response to TNF family molecules. The cells that consistently demonstrated susceptibility to TNFα and LTα were oligodendrocytes, whereas astrocytes, microglial cells and neurones showed rather protective or proliferative responses (Selmaj *et al.*, 1991; Selmaj and Raine, 1998). In contrast to the TRAIL killing of oligodendrocytes reported by Nitsch and colleagues (Nitsch *et al.*, 2000), the kinetics of TRAIL-induced oligodendrocyte apoptosis in our experimental system with purified glial cell culture was similar to that reported previously for TNFα and LTα (Hisahara *et al.*, 1997), where oligodendrocytes were killed 48–72 h post-exposure to TRAIL.

The differential sensitivity of a wide variety of cell types to TRAIL has been attributed to differences in the expression of TRAIL-Rs (Griffith and Lynch, 1998; Zhang *et al.*, 2000*a*). TRAIL mediates apoptotic cell death by interaction with two distinct receptors containing death domains, TRAIL-R1 and TRAIL-R2 (Pan *et al.*, 1997; Walczak *et al.*, 1997). However, TRAIL-R1 and TRAIL-R2 are widely expressed on most cell types and, therefore, the alternative hypothesis is that the non-signalling TRAIL-Rs, TRAIL-R3 and TRAIL-R4, act as decoy receptors and determine whether a cell is resistant or sensitive to TRAIL-induced cell death (Degli-Esposti *et al.*, 1997; Sheridan *et al.*, 1997).

Our results showed that both TRAIL-R1 and TRAIL-R2 are expressed on oligodendrocytes and microglial cells to the same level. However, the blocking experiments demonstrated that TRAIL-R1 is responsible for mediating the death signal in ahOL. In most of the transformed cells, TRAIL-induced death is mediated by TRAIL-R2 (MacFarlane *et al.*, 1997). The pattern of immunoreactivity of TRAIL-R2 in western blotting differed between ahOL and most of the control-transformed cell lines, which showed two bands instead of one band. This might suggest that, in normal cells, TRAIL-R2 is less prone to mediate the death signal and that TRAIL-R1 is the primary death mediating receptor of the TRAIL pathway. Such selective oligodendrocyte sensitivity to TRAIL may be related to a differential expression of the decoy receptors on these cells. Consistent with this hypothesis is our observation that microglial cells, which are resistant to TRAIL-induced cell death, expressed high levels of decoy TRAIL-R3 whereas oligodendrocytes and the transformed cell lines, MO3.13 and glioblastoma, expressed low levels of TRAIL-R3 or none at all. The differential expression of TRAIL-R3 was evident with flow cytometry, but not from the western blot analysis. This discrepancy could be explained by impaired translocation of TRAIL-R3 from the cytosol to the surface in oligodendrocytes and transformed cells. Decoy receptors are predominantly located within the cells in the nucleus, and their location within the cell suggested that expression of the receptors may involve regulated movement from intracellular compartments to the membrane (Zhang *et al.*, 2000*b*). The translocation of decoy receptors to the cell surface depends upon a signal from TRAIL-R1 and TRAIL-R2. This was in our study in which TRAIL stimulation led to enhanced TRAIL-R3 expression by microglia. However, the translocation of TRAIL-R3 did not occur in ahOL. The mechanism of the failure to effectively translocate TRAIL-R3 to the surface of ahOL is not known, but could be responsible for ahOL sensitivity to TRAIL-induced death. In support of this notion, the levels of mRNA encoding TRAIL-R1 and TRAIL-R2 were equally high in ahOL and microglia, but the mRNA for TRAIL-R3 was much higher in microglia. This suggests increased metabolic turnover of this decoy receptor in cells resistant to TRAIL-induced death. The mRNA for the other decoy receptor, TRAIL-R4, was not detected in the studied cell populations. This was in agreement with previous reports showing low and inconsistent expression of mRNA for this receptor (Griffith *et al.*, 1998).

In conclusion, we have shown for the first time that (i) TRAIL-Rs are expressed on human adult glial cells and (ii) TRAIL can induce selective ahOL apoptotic cell death which is dependent on protein synthesis inhibition and ligation of TRAIL-R1. The selectivity of TRAIL susceptibility of ahOL seems to be relevant to deficient expression of the decoy receptor TRAIL-R3. These results may be relevant to the CNS inflammatory/demyelinating conditions where the demise of oligodendrocytes occurs and may contribute to the development of new molecules that interfere with the immunopathogenesis of these diseases.

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Antileukemic drugs increase death receptor 5 levels and enhance Apo-2L–induced apoptosis of human acute leukemia cells

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In present studies, treatment with tumor necrosis factor (TNF)–related apoptosis inducing ligand (TRAIL, also known as Apo-2 ligand [Apo-2L]) is shown to induce apoptosis of the human acute leukemia HL-60, U937, and Jurkat cells in a dose-dependent manner, with the maximum effect seen following treatment of Jurkat cells with 0.25 μg/mL of Apo-2L (95.0% ± 3.5% of apoptotic cells). Susceptibility of these acute leukemia cell types, which are known to lack $p53^{wt}$ function, did not appear to correlate with the levels of the apoptosis-signaling death receptors (DRs) of Apo-2L, ie, DR4 and DR5; decoy receptors (DcR1 and 2); FLAME-1 (cFLIP); or proteins in the inhibitors of apoptosis proteins (IAP) family. Apo-2L–induced apoptosis was associated with the processing of caspase-8, Bid, and the cytosolic accumulation of cytochrome c as well as the processing of caspase-9 and caspase-3. Apo-2L–induced apoptosis was significantly inhibited in HL-60 cells that overexpressed Bcl-2 or Bcl-x_L. Cotreatment with either a caspase-8 or a caspase-9 inhibitor suppressed Apo-2L–induced apoptosis. Treatment of human leukemic cells with etoposide, Ara-C, or doxorubicin increased DR5 but not DR4, Fas, DcR1, DcR2, Fas ligand, or Apo-2L levels. Importantly, sequential treatment of HL-60 cells with etoposide, Ara-C, or doxorubicin followed by Apo-2L induced significantly more apoptosis than treatment with Apo-2L, etoposide, doxorubicin, or Ara-C alone, or cotreatment with Apo-2L and the antileukemic drugs, or treatment with the reverse sequence of Apo-2L followed by one of the antileukemic drugs. These findings indicate that treatment with etoposide, Ara-C, or doxorubicin up-regulates DR5 levels in a p53-independent manner and sensitizes human acute leukemia cells to Apo-2L–induced apoptosis. (Blood. 2000;96:3900-3906)

Introduction

Tumor necrosis factor (TNF)–related apoptosis-inducing ligand (TRAIL), also called Apo-2 ligand (Apo-2L), is a member of the TNF family, which has been shown to induce apoptosis of a variety of tumor cell lines more efficiently than normal cells.[1-3] While in a recent report, TRAIL was demonstrated to induce apoptosis of human hepatocytes, it has also been shown to actively suppress human mammary adenocarcinoma growth in mice without any significant toxic effects, which are seen with the in vivo use of TNF and Fas ligand (CD95L).[3,4] Apo-2L can bind to several members of the TNF receptor family, ie, death receptors (DRs) 4 and 5, decoy receptors (DcRs) 1 and 2, and osteoprotegerin.[1] DR4 and DR5 contain a cytoplasmic region consisting of a stretch of 80 amino acids, designated the death domain (DD), responsible for transducing the death signal.[1] Ligation by Apo-2L recruits the adaptor molecule FADD to the DD of DR4 and DR5.[5] Through its death effector domain, FADD interacts with caspase-8 and caspase-10.[5,6] Although $FADD^{-/-}$ cells have been shown to be sensitive to apoptosis induced by the ligation of DR4 and DR5 but not of Fas,[7] both caspase-8 and FADD are essential to the function of TRAIL-mediated death-inducing signaling complex (DISC).[5] Once recruited to FADD, caspase-8 drives its auto-activation through oligomerization and subsequently activates the downstream effector caspases, such as caspase-3, caspase-6, and caspase-7.[6,8] Activated and processed caspase-8 can also cleave and activate the BH3 domain containing pro-apoptotic molecule Bid, which then translocates to the mitochondria triggering the pre-apoptotic mitochondrial events, including the cytosolic release of cytochrome (cyt) c.[9-11] In the cytosol, cyt c and deoxyadenosine triphosphate (dATP) bind to Apaf-1 and cause its oligomerization.[12,13] Apaf-1, in turn, binds and processes procaspase-9 into an active caspase that recruits, cleaves, and activates the effector caspase-3.[12-14] Activated caspase-3 can proteolytically cleave a number of cellular proteins, eg, poly(ADP-ribose) polymerase (PARP), lamins, DFF 45 (ICAD, DNA fragmentation factor), fodrin, gelsolin, PKCδ, Rb, and DNA-PK, resulting in the morphologic features and DNA fragmentation of apoptosis.[6,8,15] Thus, Apo-2L–induced caspase-3 activation may occur either directly through the activity of caspase-8 and/or through Apaf-1–mediated activity of caspase-9. This is supported by the observation that while $Apaf^{-/-}$ cells are sensitive to Fas L and TNFα, they are relatively resistant to Apo-2L–induced apoptotic signaling.[16]

There are several known determinants of Apo-2L–induced apoptotic signaling. Treatment with DNA-damaging anticancer agents can induce p53 and/or NFkB, which, in turn, can up-regulate DR5 and/or DR4 expression, thereby enhancing Apo-2L–induced apoptotic signaling.[17,18] In contrast, DcR1, which is bound to the cell membrane through a glycolipid anchor and lacks DD, and the levels of DcR2, which has an incomplete and inactive DD, bind and titrate down Apo-2L and can act as inhibitors of Apo-2L–induced apoptosis.[1] Additionally, an endogenous intracellular protein,

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FLAME-1 (also known as cFLIP, CASH, CLARP, MRIT, I-FLICE, and Usurpin), which has an N-terminus FADD homology and C-terminus caspase homology domains without caspase activity, has a dominant-negative effect against caspase-8 and caspase-10 and can potentially inhibit Apo-2L–induced death signaling.[19] Finally, the levels of inhibitors of apoptosis proteins (IAP) family members, which include cIAP1, cIAP2, XIAP, and survivin, may also inhibit Apo-2L–induced apoptosis by specifically binding to and inhibiting the activities of caspase-3, caspase-9, and caspase-7.[20-22] Although the ability of Apo-2L to induce apoptosis has been examined in a variety of human tumor cell types, the molecular steps of Apo-2L–induced apoptosis and its determinants have not been comprehensively evaluated in the human acute leukemia cells.

Etoposide, Ara-C, and doxorubicin are highly active antileukemic drugs.[23] Intracellularly, following their interaction with DNA, these drugs ultimately cause DNA damage and cell-cycle arrest.[24] By, as yet, undefined signal(s), this drug-induced DNA-damage and/or cell-cycle perturbation triggers the mitochondrial $\Delta\Psi m$ and release of cyt c, which ultimately results in the activities of caspase-9 and caspase-3. Recently, etoposide, CPT-11, doxorubicin, 5-FU, and, to a lesser extent, taxol were shown to augment Apo-2L–induced apoptosis of epithelial cancer cells.[18,25-28] However, neither the molecular determinants of Apo-2L–induced apoptosis nor the interaction between chemotherapeutic agents and Apo-2L had been examined in human acute leukemia cells. In the present studies, our findings demonstrate that Apo-2L treatment triggers the processing of caspase-8 and Bid, and also causes cytosolic accumulation of cyt c, followed by Apaf-1–mediated caspase-9 and caspase-3 processing and apoptosis of human acute leukemia cells. Furthermore, our results show that treatment with etoposide, Ara-C, or doxorubicin increases DR5 levels and enhances Apo-2L–induced apoptosis of acute leukemia HL-60, U937, and Jurkat cells, which are known to lack $p53^{wt}$ function.

Materials and methods

Reagents

We purchased z-IETD-fmk and z-LEHD-fmk from Enzyme Systems Products (Livermore, CA). Anti–Apaf-1 and anti-Bid antiserums[9,13] were kindly provided by Dr Xiaodong Wang of the University of Texas, Southwestern School of Medicine (Dallas). The recombinant human homotrimeric TRAIL (Apo-2L) (leucine zipper construct) was a gift from Immunex Corp (Seattle, WA).[4] Fas receptor (CD95) and ligand (FasL) monoclonal antibodies were purchased from Transduction Labs (Lexington, KY). Monoclonal anti-XIAP antibody was purchased from Boehringer Mannheim (Indianapolis, IN), while anti–cIAP-1 antibody was purchased from Pharmingen Inc (San Diego, CA). Polyclonal anti-DR4, anti-DcR1 and anti-DcR2, and anti–Apo-2L antibodies as well as Apo-2L R2 (DR5):Fc were purchased from Alexis Corp (San Diego, CA). Polyclonal anti-DR5 was obtained from Cayman Chemicals Co (Ann Arbor, MI).

Cell culture

Human acute leukemia HL-60, HL-60/Bcl-2, HL-60/Bcl-x_L, U937, and Jurkat cells were maintained in a humidified 5.0 % CO_2 environment in RPMI medium supplemented with 100 U penicillin per milliliter, 100 μg streptomycin per milliliter, 1% nonessential amino acids, 1% essential amino acids, and 10% bovine calf serum (Gibco Laboratories, Grand Island, NY), as previously described.[29]

Preparation of S-100 fraction and Western analysis of cytosolic cytochrome c

Untreated and drug-treated cells were harvested by centrifugation at 1000*g* for 10 minutes at 4°C. The cell pellets were washed once with ice-cold phosphate-buffered saline (PBS) and resuspended with 5 vol buffer (20 mmol/L Hepes-KOH, pH 7.5, 10 mmol/L KCl, 1.5 mmol/L $MgCl_2$, 1 mmol/L sodium EDTA, 1 mmol/L sodium EGTA [ethylene glycol-bis {B-amino ethyl ether} N,N,N',N-tetra acetic acid], 1 mmol/L dithiothreitol, and 0.1 mmol/L phenylmethylsulfonyl fluoride), containing 250 mmol/L sucrose. The cells were homogenized with a 22-gauge needle, and the homogenates were centrifuged at 100 000*g* for 30 minutes at 4°C (S-100 fraction).[12,30] The supernatants were collected, and the protein concentrations of S-100 were determined by the Bradford method (Bio-Rad, Hercules, CA). We used 20 to 30 μg of the S-100 fraction for Western blot analysis of cyt c, as described previously.[31,32]

Western analyses of proteins

Western analyses of DR4, DR5, Apo-2L, caspase-8, caspase-9, caspase-3, Fas R, Fas L, Bid, PARP, XIAP, cIAP, survivin, and β-actin were performed with the use of specific antiserums or monoclonal antibodies according to previously reported protocols.[31,32] Horizontal scanning densitometry was performed on Western blots by acquisition into Adobe PhotoShop (Apple, Cupertino, CA) and analysis by the NIH Image Program (US National Institutes of Health, Bethesda, MD). The expression of β-actin was used as a control.

Apoptosis assessment by Annexin-V staining

After drug treatments, cells were resuspended in 100 μL staining solution (containing Annexin-V fluorescein and propidium iodide Annexin-V-FLUOS Staining Kit buffer, Boehringer Mannheim). Following incubation at room temperature for 15 minutes, cells were analyzed by flow cytometry.[29] Annexin V binds to cells that express phosphotidylserine on the outer layer of the cell membrane, and propidium iodide stains the cellular DNA of cells with a compromised cell membrane. This allows for the discrimination of live cells (unstained with either fluorochrome) from apoptotic cells (stained only with Annexin V) and necrotic cells (stained with both Annexin V and propidium iodide).[33]

Morphology of apoptotic cells

After drug treatment, 50×10^3 cells were washed with PBS (pH 7.3) and resuspended in the same buffer. Cytospin preparations of the cell suspensions were fixed and stained with Wright stain. Cell morphology was determined by light microscopy. In all, 5 different fields were randomly selected for counting of at least 500 cells. The percentage of apoptotic cells was calculated for each experiment, as described previously.[34]

Statistical analysis

Significant differences between values obtained in a population of leukemic cells treated with different experimental conditions were determined by paired *t*-test analyses. A one-way analysis of variance was also applied to the results of the various treatment groups, and post hoc analysis was performed by means of the Bonferroni correction method.

Results

Apo-2L induces apoptosis of human acute leukemia, HL-60, U937, and Jurkat cells

Although Apo-2L has been reported to induce apoptosis of a variety of tumor cell types,[3,4] the sensitivity of human acute leukemia cells to Apo-2L–induced apoptosis and its molecular determinants had not been comprehensively determined. The results of present studies (Figure 1A) demonstrate that exposure to Apo-2L for 24

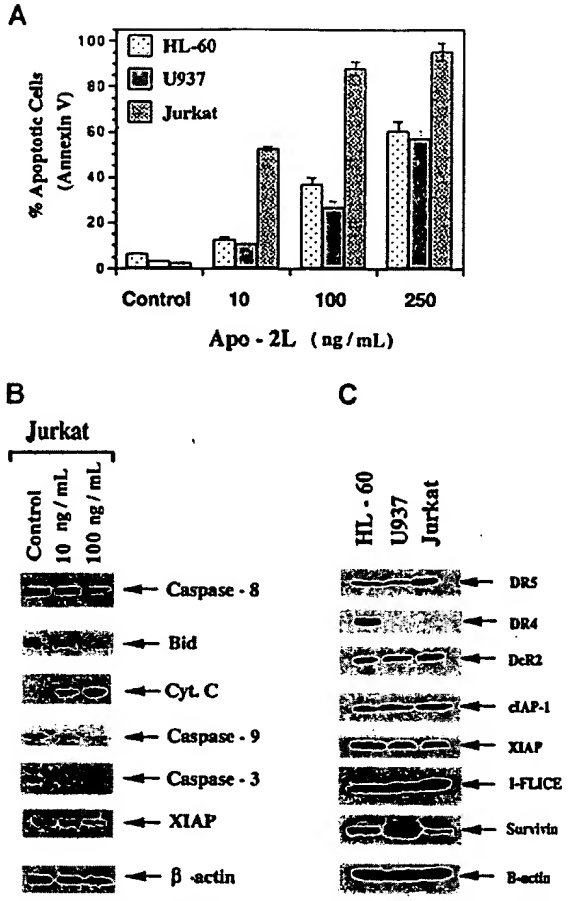

Figure 1. Apo-2L induces apoptosis of HL-60, U937, and Jurkat cells. Cells were exposed to the indicated concentrations of Apo-2L for 24 hours (A). Following this, the percentage of apoptotic cells were determined (see "Results"). Apo-2L induced the processing and down-regulation of caspase-8, Bid, caspase-9, caspase-3, and XIAP, as well as cytosolic accumulation of cyt c in Jurkat cells (B). Following exposure of Jurkat cells to 100 ng/mL of Apo-2L for 24 hours, cell lysates were obtained and Western analyses of the indicated proteins were performed (see "Results"). Western analyses of DR4, DR5, DcR2, cIAP-1, XIAP, I-FLICE (FLAME-1 or cFLIP), and survivin in HL-60, U937, and Jurkat cells (C).

hours induced a dose-dependent increase in apoptosis of the cultured acute leukemia HL-60, U937, and Jurkat cells, as determined by Annexin V staining followed by flow cytometry. This was confirmed by light-microscopic morphologic examination of the Wright-stained, cytospun, Apo-2L–treated cells (data not shown). Jurkat cells demonstrated the highest sensitivity: exposure to 250 ng/mL of Apo-2L induced apoptosis of 95.0% ± 3.5% of the cells. Treatment with 100 ng/mL of Apo-2L also produced significantly more apoptosis of Jurkat, followed by HL-60 and U937 cells ($P < .01$). As shown in Figure 1B, treatment of Jurkat cells with Apo-2L was associated with the processing of caspase-8 and Bid, as well as the cytosolic accumulation of cyt c. Exposure to Apo-2L also resulted in the processing of caspase-9 and caspase-3 and down-regulation of XIAP. In these immunoblots, with the commercially available antibodies, we have not been able to uniformly detect the cleaved fragments of the processed pro-forms of caspase-8, caspase-9, and caspase-3. With processing, the levels of the pro-forms decline, as shown in the immunoblots. These effects were more pronounced after treatment of Jurkat cells with 100 ng/mL Apo-2L, as compared with 10 ng/mL (Figure 1B). Exposure to Apo-2L also induced similar molecular events in HL-60 and U937 cells (data not shown). Thus, in the acute leukemia cells, Apo-2L also triggered the intrinsic pathway of apoptosis. Some but not all previous reports had shown a positive correlation of the sensitivity to Apo-2L with the expression of DR4 and DR5, or with the intracellular levels of FLAME-1.[3,4,27,28] In contrast, as also previously reported for other cell types,[3,18,27] data presented in Figure 1C do not show such a correlation in the leukemic cells. As compared with other cell types, Jurkat cells, which demonstrated the highest sensitivity to Apo-2L, expressed higher levels of DR5, but lacked the expression of DR4 (Figure 1C). Inconsistent with their increased sensitivity to Apo-2L, Jurkat cells expressed more DcR2 and FLAME-1 (I-FLICE) although their survivin levels were the lowest of the 3 cell types (Figure 1C). All cell types expressed barely detectable levels of DcR1 (data not shown).

Apo-2L–induced apoptosis of leukemic cells was inhibited by overexpression of Bcl-2 or Bcl-x_L

We examined the effect of Bcl-2 or Bcl-x_L overexpression on Apo-2L–induced apoptosis of HL-60 cells. As compared with HL-60/neo cells, HL-60/Bcl-2 and HL-60/Bcl-x_L cells possess approximately 3-fold higher levels of Bcl-2 and 5-fold higher levels of Bcl-x_L, respectively (Figure 2B).[35] Exposure to 100 ng/mL of Apo-2L induced apoptosis of 37.0% ± 2.0% of HL-60/neo cells. However, Apo-2L–induced apoptosis was suppressed in HL-60/Bcl-2 and HL-60/Bcl-x_L cells (Figure 2A). This supported the observation that, in HL-60 cells, Apo-2L triggered the intrinsic pathway of apoptosis. This conclusion was further supported by the finding that cotreatment with the relatively specific caspase-9 inhibitor z-LEHD-fmk was as effective as the caspase-8 inhibitor z-IETD-fmk in suppressing Apo-2L–induced apoptosis of HL-60 cells (Figure 2C).

Etoposide-, Ara-C–, or doxorubicin-induced apoptosis is associated with up-regulation of DR5 but not DR4, Fas, or DcR1 and DcR2

Etoposide, Ara-C, and doxorubicin are commonly used antileukemic drugs. With the goal of preclinically investigating the antileukemic activity of novel combinations of Apo-2L with relatively high but clinically deliverable doses of etoposide, Ara-C, or doxorubicin, we first determined the sensitivity and molecular cascade of apoptosis triggered by these drugs in HL-60, U937, and Jurkat cells. Figure 3A clearly demonstrates that high but clinically achievable and relevant doses of etoposide, Ara-C, and doxorubicin induced apoptosis of approximately 30% to 75% of the leukemic cells. As also previously reported by us,[35,36] these drugs triggered the intrinsic pathway of apoptosis by inducing the cytosolic accumulation of cyt c as well as the processing of caspase-9 and caspase-3 (Figure 3B). Treatment with etoposide, Ara-C, and doxorubicin (not shown) was also associated with down-regulation of XIAP and survivin levels (Figure 3C). XIAP has been previously reported to be processed during Fas-mediated apoptosis,[37] while survivin expression is cell-cycle phase–dependent and is down-regulated during the nonmitotic phases.[38] Importantly, treatment with etoposide, Ara-C, or doxorubicin induced DR5 levels in HL-60 and Jurkat cells (Figure 4A). This was also observed to a lesser extent in U937 cells (data not shown). In contrast, DcR2, Apo-2L, FasL, and Fas levels remained unaffected (Figure 4A). In all cell lines, DcR1 levels were barely detectable (data not shown). Figure 4B-C shows that exposure of HL-60 cells to 1.0 μmol/L or higher Ara-C for 6 hours or longer was necessary to produce an

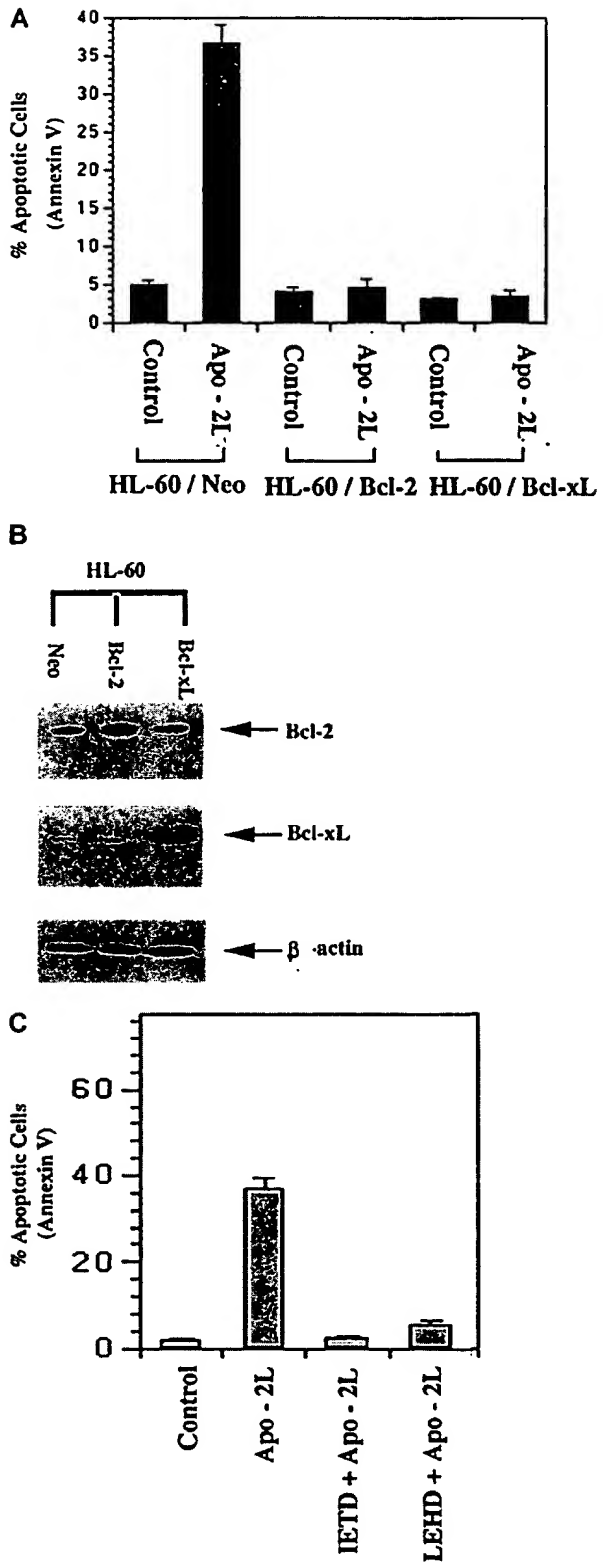

Figure 2. Overexpression of Bcl-2 or Bcl-x_L inhibited Apo-2L–induced apoptosis of HL-60 cells (A). Following treatment with 100 ng/mL of Apo-2L, the percentage of apoptotic cells was determined by Annexin V staining and flow cytometry (A). Western blot analyses of Bcl-2 and Bcl-x_L levels demonstrating overexpression of Bcl-2 and Bcl-x_L in HL-60/Bcl-2 and HL-60/ Bcl-x_L cells, respectively. Levels of β-actin served as the loading control (B). Cotreatment with the relatively specific caspase-8 inhibitor (z-IETD-fmk, 50 μmol/L) or caspase-9 inhibitor (z-LEHD-fmk, 100 μmol/L) inhibited Apo-2L–induced apoptosis (C).

increase in DR5 levels. Lower levels and shorter exposure intervals to Ara-C did not increase DR5 levels in any cell type (data not shown). Although not shown, treatment with Ara-C, while markedly increasing DR5, reduced DR4 levels in HL-60 cells. This was not observed in the other cell types, which express very low levels of DR4 (data not shown).

Although treatment with the antileukemic drugs did not increase Apo-2L expression, to confirm that etoposide- or Ara-C–induced apoptosis was not mediated by even a transient induction of Apo-2L and that triggering of DR5-mediated apoptosis, we compared the effect of cotreatment with Apo-2L-R2(DR5):Fc on apoptosis induced by the antileukemic drugs or by Apo-2L. If treatment with the antileukemic drug produced any Apo-2L in the culture medium of the cells, apoptosis triggered by this, through induced DR5, would be blocked by Apo-2L-R2. As shown in Figure 4D, cotreatment with Apo-2L-R2:Fc (20 ng/mL) inhibited Apo-2L– but not etoposide- or Ara-C–induced apoptosis of HL-60 cells.

Pretreatment with the antileukemic drugs increases Apo-2L–induced apoptosis

To determine the functional significance of DR5 induction by the antileukemic drugs, we compared the apoptotic effects of the sequential treatment with Ara-C, etoposide, or doxorubicin followed by Apo-2L (6 hours of the drug followed by drug washout and 18 hours of Apo-2L treatment) with those of the drug administered alone or together with Apo-2L. Figure 5 demonstrates that significantly more apoptosis was observed following a sequential treatment of HL-60 cells with etoposide or Ara-C followed by Apo-2L, as compared with treatment with Apo-2L or either of the drugs alone. Sequential treatment with the drug followed by Apo-2L also yielded more apoptosis than cotreatment with Apo-2L plus Ara-C or etoposide ($P < .01$). To adequately assess the potentiating effects of pretreatment with the antileukemic drugs on Apo-2L–induced apoptosis, relatively lower concentrations of etoposide or Ara-C were used for these studies. Although not shown, a sequential treatment with Ara-C or etoposide followed by Apo-2L was also more effective than treatment with the reverse sequence of Apo-2L followed by either of the 2 drugs. For example, exposure to the reverse sequence of Apo-2L (100 ng/mL) for 18 hours followed by Ara-C (10 μmol/L for 6 hours) produced apoptosis of only 47% ± 6% of cells, as compared with apoptosis of 88% ± 9% cells observed with treatment with Ara-C followed by Apo-2L. Similar observations were made when doxorubicin was administered with Apo-2L, and Jurkat cells were used to investigate these treatment schedules (data not shown).

Discussion

Several reports have demonstrated the sensitivity and molecular correlates of Apo-2L–induced apoptosis of cancer cells.[3,4,25-28] Some of these reports have also shown that chemotherapeutic agents increase Apo-2L–induced apoptosis of epithelial cancer cells.[25-28] In the present studies, however, we demonstrate for the first time that Apo-2L signals apoptosis of acute leukemia HL-60, U937, and Jurkat cells mainly through the intrinsic mitochondrial pathway of apoptosis. Since these cells lack a functional $p53^{wt}$, our data also indicate that Apo-2L–induced apoptosis is independent of p53 status. This has also been demonstrated for other tumor cell

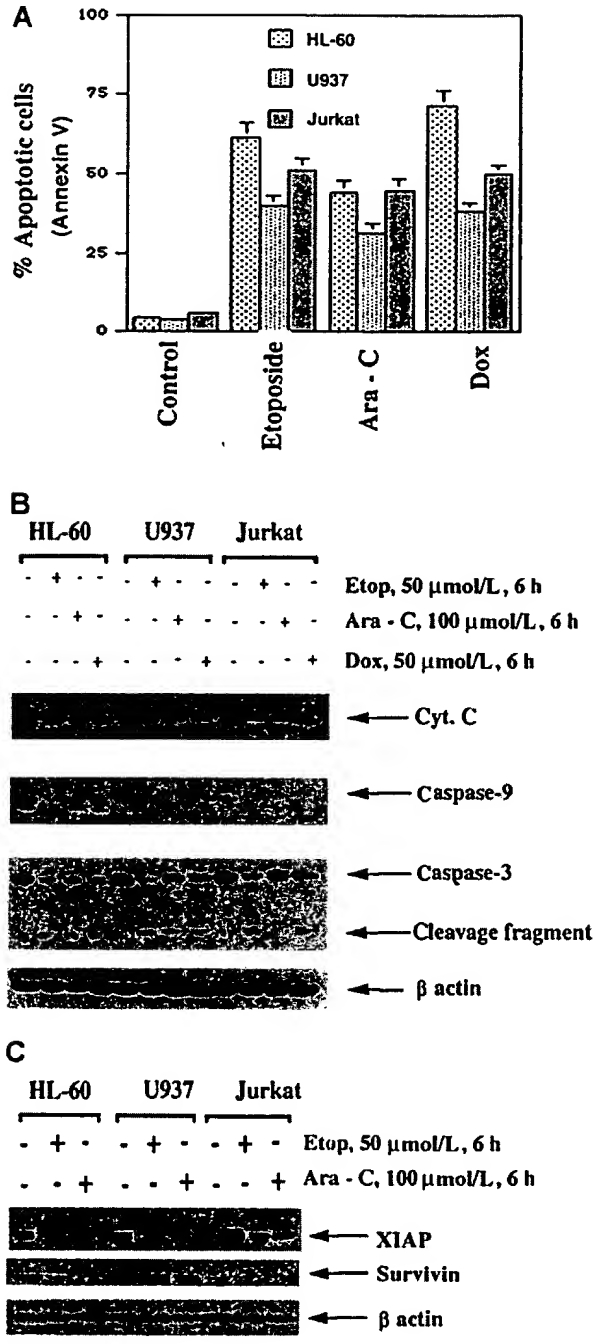

Figure 3. Etoposide (50 µmol/L), Ara-C (100 µmol/L), and doxorubicin (50 µmol/L) induced apoptosis of HL-60/neo, U937, and Jurkat cells. Cells were treated with the drugs at the indicated concentrations for 6 hours followed by incubation for 18 hours in drug-free medium. After these treatments, the percentage of apoptotic cells was determined by Annexin V staining followed by flow cytometry (A). Molecular events of apoptosis induced by etoposide, Ara-C, or doxorubicin (B). Cells were treated with the indicated drugs for 6 hours, and then cell lysates were obtained for Western analyses of procaspase-3, procaspase-9, cytosolic cyt c, and Bid protein. Alternatively, Western analysis of XIAP and survivin was performed on the cell lysates (C) (see "Results").

types.[27] Although the Apo-2L–sensitive acute leukemia cell types studied here expressed DR5 and, in HL-60, also DR4, the level of expression of these death-signaling receptors did not correlate with the sensitivity to Apo-2L. This correlation has been demonstrated by some but not all previously reported studies.[3,25,27] DcR1 and DcR2 do not transduce Apo-2L–induced death signal.[1,2] DcR2 has also been shown to inhibit apoptotic signaling by inducing NFkB activity.[39] Ectopic overexpression of DcR1 and DcR2 has been shown to inhibit Apo-2L–induced apoptosis.[1,40] However, in the present studies, the level of expression of the decoy receptors did not correlate with resistance to Apo-2L–induced apoptosis of the leukemic cells. These findings are similar to other previously reported observations about melanoma and breast cancer cells.[3,25,41] We also did not find any correlation between intracellular FLAME-1 (cFLIP or I-FLICE) levels and the sensitivity of the leukemic cells to Apo-2L–induced apoptosis. The spliced variants of cFLIP are the long form ($cFLIP_L$) and short form ($cFLIP_S$). $cFLIP_L$, which was the variant detected in our immunoblots by the antibody used, has the inhibitory effect on Fas L- and TRAIL-induced DISC activity.[41] Although, similarly to our findings, increased levels of cFLIP have been shown to inhibit apoptosis owing to death receptor signals and have been correlated with resistance to Apo-2L–induced apoptosis,[27,42-45] this has not been observed in all cell types.[3] Since FLAME-1 exerts its inhibitory effect by binding to FADD and caspase-8, this dominant-negative effect on the activity of the DISC may depend on the levels and role of FADD in mediating Apo-2L–induced apoptosis.[5]

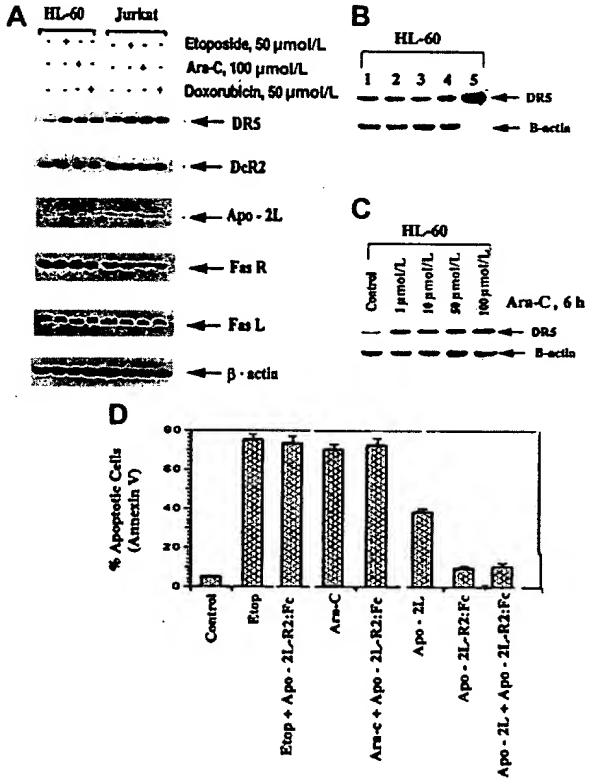

Figure 4. Etoposide, Ara-C, and doxorubicin increase DR5 levels in HL-60 and Jurkat cells (A). Following exposures of HL-60 and Jurkat cells with the indicated drug concentrations, DR5, DcR2, Apo-2L, Fas, Fas L, and β-actin levels were determined by immunoblot analyses (see "Results"). Effect of exposure intervals to Ara-C on DR5 levels of HL-60 cells (B). HL-60 cells were exposed to 1.0 µmol/L Ara-C for 6 hours. Cell lysate of untreated (lane 1), or drug-treated cells after 2 (lane 2), 4 (lane 3), 6 (lane 4), and 24 hours (of which 18 hours were in drug-free medium) (lane 5) were subjected to immunoblot analysis of DR5 levels (see "Results"). Levels of β-actin were used as the loading control. Effect of the dose of Ara-C on DR5 levels (C). HL-60 cells were exposed to different concentrations of Ara-C for 6 hours. Following this, immunoblot analysis of DR5 levels was performed; β-actin levels were used as the loading control.

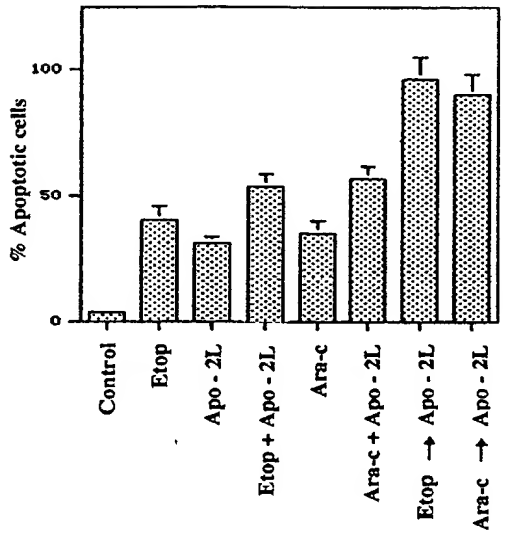

Figure 5. Pretreatment with etoposide or Ara-C enhances Apo-2L–induced apoptosis of HL-60 cells. HL-60 cells were exposed to etoposide (5.0 μmol/L, 6 hours), Ara-C (10.0 μmol/L, 6 hours), or Apo-2L (100 ng/mL, 18 hours), and the percentage of apoptotic cells was determined at the end of 24 hours (see "Results"). Cells were also exposed to etoposide plus Apo-2L (total 24 hours), or Ara-C plus Apo-2L (total 24 hours). Alternatively, cells were treated with etoposide (5.0 μmol/L, 6 hours), followed by Apo-2L (100 ng/mL, 18 hours) or Ara-C (10.0 μmol/L, 6 hours), followed by Apo-2L (100 ng/mL, 18 hours). Following these treatments, the percentage of apoptotic cells was determined by Annexin V staining and flow cytometry (see "Results").

Treatment of the acute leukemia cells with Apo-2L clearly resulted in the processing of caspase-8, Bid, caspase-9, and caspase-3, as well as the cytosolic accumulation of cyt c. This indicates that Apo-2L–induced processing and activation of caspase-8 triggered caspase-3 activity through the intrinsic (mitochondrial) pathway of apoptosis. Bcl-2 and Bcl-x_L overexpression have been previously shown to exert their inhibitory effect on apoptosis by blocking the release of cyt c and mitochondrial $\Delta\Psi m$ induced by a variety of apoptotic stimuli that engage the intrinsic pathway to apoptosis.[35,36] Accordingly, present studies demonstrate that overexpression of Bcl-2 and Bcl-x_L inhibited Apo-2L–induced apoptosis of HL-60 cells. Keogh et al[46] have recently reported that in CEM cells with known overexpression of Bcl-2, UV- but not TRAIL-induced cytosolic accumulation of cyt c and apoptosis was blocked. However, in this report they did not show Bcl-2 overexpression, raising the possibility that Bcl-2 expression was relatively low. The conclusion that Apo-2L predominately engages the intrinsic pathway of apoptosis in these cells is also confirmed by our observation that the inhibition of caspase-9 activity by z-LEHD-fmk was as effective as the inhibition of caspase-8 by z-ITED-fmk in suppressing Apo-2L–induced apoptosis of HL-60 cells. These findings suggest that acute leukemia HL-60 cells may be of the type II variety with respect to the death receptor–initiated apoptotic signaling, as was previously found for CD95 signaling.[47] Apo-2L–induced activity of the effector caspases, such as caspase-3, was also associated with down-regulation of XIAP levels. As has been previously reported, this may be due to XIAP processing by caspase-3.[37]

Previous studies and data presented here demonstrate that antileukemic drugs that cause DNA damage, eg, etoposide, Ara-C, and doxorubicin, also trigger the mitochondrial or intrinsic pathway of apoptosis.[16,34-36] The resulting cytosolic accumulation of cyt c produces Apaf-1–, dATP-, and caspase-9–mediated activation of caspase-3,[34-36] all of which can be inhibited by overexpression of Bcl-2 or Bcl-x_L.[34-36] In the present studies, we have extended these observations and demonstrated that etoposide- and Ara-C–induced caspase activity and apoptosis are also associated with down-regulation of the levels of XIAP and survivin. Although survivin has not been shown to be processed by caspases, its levels are low in nonmitotic phases of the cell cycle and increase markedly during mitosis.[38] Since treatment with relatively high but clinically achievable and relevant doses of Ara-C, etoposide, or doxorubicin, as employed in the present studies, is known to arrest and increase the percentage of acute leukemia cells in the premitotic phases (G_1, S, or G_2) of the cell cycle, this may lower survivin expression in the drug-treated cells. Recent studies have suggested that chemotherapeutic agents might trigger apoptosis by inducing Fas or Fas L and activating the Fas-dependent pathway to apoptosis.[48-50] However, other reports have shown that chemotherapeutic agents induce apoptosis through a Fas-independent pathway of apoptosis.[51,52] Data from present studies also support this by demonstrating that etoposide, Ara-C, and doxorubicin did not induce Fas or Fas L. Treatment with these drugs also did not increase the expression of Apo-2L. Furthermore, cotreatment with a fusion protein containing the extracellular domains of DR4 or DR5 fused to the immunoglobin Fc region, ie, Apo-2L R1, or R2:Fc, did not inhibit etoposide- or Ara-C–induced, but blocked Apo-2L–induced, apoptosis. These data make it unlikely that FasL or Apo-2L expression is induced by etoposide or Ara-C or that the ligation of DR5 in the acute leukemic cells plays any significant role in apoptosis induced by these antileukemic drugs.

Although Apo-2L expression was not enhanced, treatment with etoposide, Ara-C, and doxorubicin increased DR5 but not DR4 levels in the 3 acute leukemia cell types. This was observed after treatment with a threshold concentration and an exposure interval of each of the drugs. As noted above, these concentrations of the drugs are clinically achievable during the administration of induction therapy in relapsed leukemias with relatively high doses of these drugs. Previous reports have implicated p53 and NFkB as the transactivating factors in DR5 and DR4 up-regulation by DNA-damaging drugs such as etoposide or doxorubicin.[18,53] Although the role of $p53^{wt}$ function in mediating DR5 up-regulation by the antileukemic drugs in the acute leukemia cells studied here can be excluded, NFkB may have been involved in mediating this effect. Regardless of the transcriptional activator involved in DR5 induction, the results presented here indicate that the increased DR5 levels due to treatment with antileukemic drugs should preferentially potentiate Apo-2L–induced apoptosis when exposure to Apo-2L follows treatment with the antileukemic drug. This was corroborated by our findings, which show that pretreatment of the leukemic cells with each of the 3 antileukemic drugs yielded more Apo-2L–induced apoptosis than treatment with Apo-2L, the drugs alone, or even cotreatment with Apo-2L plus each of the antileukemic drugs, or by the reverse sequence of exposure to Apo-2L followed by the antileukemic drugs.

In summary, although Apo-2L and each of the antileukemic drugs studied here are shown to engage the intrinsic pathway of apoptosis by up-regulating DR5 levels, pretreatment with the drugs increases the responsiveness to Apo-2L–induced apoptosis. These data suggest a strategy to rationally combine Apo-2L and conventional antileukemic drugs in a regimen that would optimize the antileukemic activity and induce apoptosis of acute leukemia cells, which lack a functional $p53^{wt}$.

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TRAIL/Apo-2 Ligand Induces Primary Plasma Cell Apoptosis[1]

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Apoptosis constitutes the primary mechanism by which noncycling plasma cells are eliminated after the secretion of Ag-specific Abs in a humoral immune response. The underlying mechanism is not known. Here, we demonstrate that the expression of both TRAIL/Apo-2 ligand and the death receptors (DR) DR5 and DR4, but not Fas, are sustained in IL-6-differentiated Ig-secreting human plasma cells as well as primary mouse plasma cells generated in a T-dependent immune response. Plasma cell apoptosis is induced by both endogenous and exogenous TRAIL ex vivo, suggesting that TRAIL-mediated killing may, in part, be plasma cell autonomous. By contrast, resting and activated B cells are resistant to TRAIL killing despite comparable expression of TRAIL and DRs. The preferential killing of plasma cells by TRAIL correlates with decreased expression of CD40 and inactivation of NF-κB. These results provide the first evidence that primary plasma cells synthesize TRAIL and are direct targets of TRAIL-mediated apoptosis, which may relate to the inactivation of the NF-κB survival pathway. ***The Journal of Immunology,*** **2002, 169: 5505–5513.**

During B cell terminal differentiation, Ag-activated B cells are either eliminated by apoptosis, due to low Ag affinity or self-reactivity, or differentiated to affinity-matured memory B cells or Ab-secreting plasma cells (1). Plasma cells are permanently withdrawn from the cell cycle and memory cells cycle infrequently, if at all. With the exception of long-lived plasma cells found in the bone marrow and the lamina propria of the intestine, most plasma cells are rapidly eliminated by cell death after the synthesis and secretion of large amounts of Abs (2–4). Cell death thus represents the primary mechanism that controls plasma cell homeostasis; however, the mechanisms of plasma cell death are not known.

The expansion of activated B cells is tightly regulated by survival and apoptotic signals mediated by CD40 and Fas, two members of the TNFR family. Likewise, the proliferation and survival of germinal center B cells in a T-dependent immune response critically depends on CD40 signaling through TNFR-associated factor (TRAF)[3] and NF-κB (5, 6). TRAIL/Apo-2 ligand (Apo2L) is a proapoptotic member of the TNF family (7, 8), which appears to predominantly induce apoptosis of tumor cells (9, 10), including lymphoma and myeloma (malignant plasma) cell lines (7, 8, 10–13). Whether TRAIL has a role in the control of B lineage cells has not been defined.

TRAIL functions to either accelerate or attenuate apoptosis depending on its interaction with five distinct receptors (DRs). After ligand binding and receptor trimerization, DR4 (TRAIL-R1) and DR5 (TRAIL-R2) recruit Fas-associated death domain-containing protein (FADD) to their cytoplasmic domains (14–17). This newly formed complex initiates an apoptotic cascade through the recruitment and activation of caspase-8 (15–17) or by a caspase-independent mechanism involving the receptor-interacting protein (RIP) (18). The other known TRAIL receptors are decoy receptor (DcR)1 (TRAIL-R3) and DcR2 (TRAIL-R4) (14), which lack intact cytoplasmic death domains, and the soluble osteoprotegerin (19). These receptors sequester TRAIL from DR4 and DR5, thereby antagonizing TRAIL-mediated apoptosis (14).

The humoral immune response requires IL-6, given that IgG and IgA responses are defective in the absence of IL-6 (20, 21). In vitro, stimulation of EBV-immortalized, IgG-bearing human lymphoblastoid cells with IL-6 recapitulates all major hallmarks of B cell terminal differentiation (22–25). These include prominent increases in Ig synthesis and secretion, extinction of surface MHC class II expression, and cell cycle arrest (22–25). Most importantly, the IL-6-differentiated plasma cells no longer exhibit a transformed phenotype due to loss of EBV-transforming gene expression and rapidly undergo apoptosis (22), thereby mimicking short-lived plasma cells. The human lymphoblastoid cells resemble CD40-activated B cells in that the TRAF signaling pathway is constitutively activated by the EBV-encoded latent membrane protein-1 (LMP1) (26, 27). The loss of LMP1 expression in IL-6-differentiated plasma cells suggests that apoptosis of plasma cells may relate to the loss of NF-κB activation and an altered balance between survival and death signals.

Here, we show that the IL-6-differentiated human plasma cells retain the expression of TRAIL, DR4, and DR5 and rapidly undergo apoptosis mediated by endogenous and exogenous TRAIL. Induction of apoptosis by TRAIL extends to primary mouse plasma cells but not resting or activated B cells. This preferential TRAIL-mediated killing correlates with loss of CD40 expression

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[3] Abbreviations used in this paper: TRAF, TNFR-associated factor; Apo2L, Apo-2 ligand; DR, death receptor; FADD, Fas-associated death domain-containing protein; DcR, decoy receptor; LMP1, latent membrane protein-1; CD40L, CD40 ligand; mTRAIL, membrane-bound murine TRAIL; NP, 4-hydroxy-3-nitrophenyl; NP-CGG, NP-chicken γ-globulin; RIP, receptor-interacting protein; ZVAD, benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone; FasL, Fas ligand.

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and NF-κB activation in plasma cells. Thus, plasma cells synthesize TRAIL and are subject to TRAIL-mediated apoptosis, which may relate to the inactivation of the CD40-NF-κB pathway.

Materials and Methods

Terminal differentiation of CESS cells by IL-6 and induction of apoptosis by TRAIL

CESS is an EBV-immortalized, IgG-bearing human lymphoblastoid B cell line (22), Jurkat cells were obtained from the American Type Culture Collection, whereas the P11 human T cell line was provided by Dr. K. Elkon. CESS cells were terminally differentiated by treatment with baculovirally expressed recombinant human IL-6 and soluble IL-6 receptor (gp80) for 4 days as previously described (23). IL-6-differentiated (IgG^{high}/surface MHC class II^{low}) cells were separated from IL-6-refractory (IgG^{low}/surface MHC class II^{high}) cells using anti-MHC class II Abs conjugated to magnetic beads (Dynal, Lake Success, NY), also as previously described (23).

When indicated, cells were cultured with 20 μM benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone (ZVAD; Enzyme System Products, Livermore, CA) and 1 μg/ml recombinant soluble TRAIL/Apo2L (28), DR5-Fc (aa 52–180 of DR5 fused to the Fc portion of human IgG) (29), both generously provided by A. Ashkenazi (Genentech, South San Francisco, CA), or the Fc portion of human IgG (Rockland, Bridgeport, NJ). Recombinant TRAIL and DR5-Fc provided by Dr. Ashkenazi were used in Figs. 3 and 6*A*. Otherwise, cells were incubated with recombinant soluble FLAG-tagged TRAIL/Apo2L (0.1 μg/ml) together with a FLAG enhancer Ab (1 μg/ml) and DR5-Fc (1 μg/ml) purchased from Alexis Biochemicals (San Diego, CA). Cell viability was determined by trypan blue exclusion.

In vitro terminal differentiation of primary mouse B cells

Resting splenic B cells were isolated from 5- to 8-wk-old BALB/c mice (The Jackson Laboratory, Bar Harbor, ME) and cultured with CD40 ligand (CD40L)-expressing L cells in the presence of human IL-6 (40 U/ml) and the soluble gp80 subunit of the IL-6R (40 U/ml) as previously described (30). From day 9 onward, the B cells were cocultured with osteoblastic MC3T3 cells (Deutsche Sammlungion Mikroorganismen und Zell Kulturen, purchased from the German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany).

Isolation of plasma cells generated in the 4-hydroxy-3-nitrophenyl-chicken γ-globulin (NP-CGG) response and induction of apoptosis by TRAIL

Splenic B cells were isolated from B6 mice (8-wk of age) 10 days after s.c. injection with 75 μg of NP-CGG as previously described (30). To enrich for syndecan-1-positive plasma cells, viable cells were isolated by Ficoll gradient centrifugation and incubated at a concentration of 10^7 cells/ml with a biotinylated rat anti-mouse syndecan-1 Ab (1/500 dilution; BD Biosciences, Franklin Lake, NJ). This was followed by a second incubation with streptavidin conjugated to magnetic beads (Miltenyi Biotec, Auburn, CA). Syndecan-1-positive cells were either plated onto CD40L-expressing L cells in the absence or presence of DR5-Fc or Fc or cultured directly onto 2PK3 cells or a stable transfectant expressing membrane-bound murine TRAIL (mTRAIL) (31). Cell viability was determined by trypan blue exclusion after 5 h in culture. Resting and activated B cells present in the syndecan-1-negative fraction were separated by Percoll (Sigma-Aldrich, St. Louis, MO) gradient centrifugation, as previously described (30) and cultured onto mTRAIL or control 2PK3 cells for 24 h. Cell viability was determined by annexin V binding and trypan blue exclusion.

Immunofluorescence microscopy and flow cytometric analysis

Immunofluorescence microscopy was performed essentially as described (23). Unless indicated, all Abs were obtained from BD Biosciences. Fas expression in CESS cells was detected by incubation with an anti-Fas mouse mAb (1/100; PanVera, Madison, WI) followed by a second incubation with a FITC-conjugated rabbit anti-mouse Ab (1/200; DAKO, Carpinteria, CA). Intracellular IgG was detected with a Rhodamine-conjugated goat anti-human IgG Ab (1/400; Cappel, Bryan, OH). Cells were counterstained with 17 mM 4′,6′-diamino-2-phenylindole dihydrochloride and visualized by fluorescence microscopy.

Flow cytometric analysis was performed as previously described (30). CESS cells were stained with the above mentioned anti-Fas, or CD40 Abs (1/100), and were revealed by a secondary incubation with a FITC-conjugated rabbit anti-mouse Ab (1/200; DAKO). In vitro-differentiated primary mouse B cells were stained with a PE-conjugated hamster anti-mouse Fas Ab (1/100) or a FITC-conjugated rat anti-mouse CD40 Ab (1/100). For three-color staining shown in Fig. 7*A*, syndecan-negative and -positive cells were stained with a PE-conjugated rat anti-mouse syndecan-1 Ab (1/200), a FITC-conjugated rat anti-mouse CD40 Ab (1/200) and a Cy-Chrome conjugated rat anti-mouse B220 Ab (BD PharMingen, San Diego, CA) (1/400). Cells were analyzed using a BD Biosciences FACSCalibur.

ELISA and ELISPOT

Detection of human IgG secreted by IL-6-differentiated CESS cells by ELISA was performed in 96-well plates coated with 1 μg of a rabbit anti-human IgG polyclonal Ab (Cappel) (30). To detect NP-specific IgG secreted by primary plasma cells, wells were coated with 2.5 μg 4-hydroxy-3-iodo-5-nitrophenylacetyl succinimide ester conjugated to BSA. Bound Ab was revealed by incubation with biotinylated goat anti-human IgG (1/20,000; Jackson ImmunoResearch Laboratories, West Grove, PA), or biotinylated goat anti-mouse IgG (1/30,000; Sigma-Aldrich) followed by a second incubation with HRP-conjugated streptavidin (1/2000; Vector, Burlingame, CA).

A secondary immune response was elicited 5 wk after the primary response by injecting tail veins with 15 μg NP-CGG in PBS. Splenic B cells were isolated 6 days later, cultured for 4 h in medium alone, with parental 2PK3 cells or mTRAIL-expressing 2PK3 cells at a 10:1 ratio. An ELISPOT was then performed in 96-well plates coated with 1 μg of a goat anti-mouse κ Ab (Southern Biotechnology Associates, Birmingham, AL) or 2.5 μg 4-hydroxy-3-iodo-5-nitrophenylacetyl succinimide ester conjugated to BSA (30).

EMSAs

EMSA was performed to analyze the NF-κB DNA binding activity using 2.6 μg of whole cell lysate and a [^{32}P]dATP-labeled H2K site as previously described (30), or to analyze the Oct-1 DNA binding activity using a [^{32}P]dATP-labeled Oct-1 probe (H2B) (5′-GATCCCAACTCTTCACCTTATT TGCATAAGCGATTCTATAG). In competition assay, an unlabeled double-stranded oligonucleotide containing the Oct site of the Eμ enhancer (5′-AATTCACCCTGTCTCATGAATATGCAAATCAGGTGAGTCTATG-3′) was used at a 280-fold molar excess.

Immunoblotting

Whole cell lysates were prepared by incubating cells in lysis buffer (250 mM NaCl, 50 mM HEPES, pH 7; 0.1% Nonidet P-40) on ice for 10 min, supernatants were clarified by centrifugation, and 10–30 μg of total protein was resolved on SDS-PAGE and transferred onto polyvinylidine difluoride membranes. Membranes were blocked in TBS-T (10 mM Tris (pH 8), 150 mM NaCl, 0.1% Tween 20) containing 5% powdered milk for 1 h followed by a 3-h incubation with one of the following Abs: mouse anti-human caspase-7 (1-1-11; 1/1000), mouse anti-human caspase-8 (1-1-40; (1/1000), rabbit anti-human caspase-3 (585R; 1/1000) (32), all provided by Dr. Y. Lazbenik; rabbit anti-human DR4 (66901N; BD Transduction Laboratories, Lexington, KY; 1/1000), rabbit anti-human DR5 (AAP-430) (1/1000; Stressgen, Vancouver, Canada), rabbit anti-human A1 (FL-175; 1/1000; Santa Cruz Biotechnology, Santa Cruz, CA), hamster anti-mouse Bcl-2 (554218; 1/1000), or mouse anti-chick α-tubulin (T9026) (1/5000; Sigma-Aldrich). Membranes were rinsed in TBS-T and incubated with biotinylated donkey anti-mouse, donkey anti-hamster, or donkey anti-rabbit Abs (1/20,000; Jackson ImmunoResearch Laboratories) for 45 min followed by a second 30-min incubation in streptavidin-HRP (1/20,000; Jackson ImmunoResearch Laboratories). To detect IgG, membranes were probed directly with a biotinylated goat anti-human IgG Ab (1/20,000) or a biotinylated goat anti-mouse IgG Ab (1/20,000), followed by a streptavidin-HRP incubation. The immunoreactive proteins were detected by chemiluminescence (ECL; Amersham, Arlington Heights, IL). For sequential blotting, membranes were stripped in 2% SDS, 62.5 mM Tris, pH 6.8, at 50°C for 20 min.

RT-PCR and RNase protection assay

CESS cell RNA was isolated by the guanidine isothiocyanate procedure as previously described (25). Total RNA from primary B lineage cells were isolated using Trizol (Life Technologies, Gaithersburg, MD). For RT-PCR analysis, reverse transcription was performed on total RNA from 3×10^5 CESS cells or 1 μg of primary cell RNA using 1 μg oligo(dT) primer and Superscript II reverse transcriptase (Life Technologies). For the PCR analysis, 1 μl of cDNA was used with 10 pmol of each of the following primers: Fas ligand (FasL), 5′-TCAGCTCTTCCACCTACAGAA-3′ and 5′-TACAACATTCTCGGTGCCTG-3′; GAPDH, 5′-CCACCCATGGCAAAT TCCATGGCA-3′ and 5′-TCTAGACGGCAGGTCAGGTCCACC-3′; mTRAIL, 5′-GGTCTCAAAGGACAAGGTG-3′ and 5′-TTAGTTAATTA

AAAAGGCTCC-3'; mouse DR5, 5'-GTCAAAGCCGAAACACTGG-3' and 5'-TCAAACGCACTGAGATCC-3'; mouse actin, 5'-AAGATCCTGACCGAGCGTGGC-3' and 5'-CTGGAAGGTGGACAGTGAGGC-3'. DNA was amplified using the following PCR conditions: FasL, 35 cycles (30 s at 94°C; 30 s at 55°C; 45 s at 72°C); GAPDH, 20 cycles (1 min at 94°C; 1 min at 62°C; 1 min at 72°C), mTRAIL: 40 cycles (1 min at 94°C; 1 min at 52°C; 2 min at 72°C); mDR5, 40 cycles (1 min at 94°C; 1 min at 52°C; 2 min at 72°C) and mouse actin, 30 cycles (1 min at 94°C; 1 min at 68°C; 2 min at 72°C) The PCR products were electrophoresed on 2% agarose gels and stained with ethidium bromide.

For the RNase protection analysis, antisense RNA was generated from the human hAPO-3c multiprobe template set (BD PharMingen) as described (33). Total RNA (5 μg) was hybridized with 1×10^6 cpm of the radiolabeled probe, subjected to RNase protection analysis, and electrophoresed on 6% denaturing acrylamide gels containing urea (33).

Results

Decreased Fas expression on IL-6-differentiated human plasma cells in vitro

IL-6-differentiated human IgG plasma cells rapidly undergo apoptosis as evidenced by annexin V binding and poly(ADP)ribose polymerase cleavage (22, 23). These plasma cells (IgG^{high}), which no longer possess a transformed phenotype due to reversal of EBV immortalization (22), can be enriched to >80% purity by negative selection for the loss of MHC class II expression (23). To verify the involvement of caspases in plasma cell death, the IL-6-differentiated plasma cells and control CESS cells were cultured in the presence or absence of the general caspase inhibitor, ZVAD (Fig. 1*A*). Although ZVAD did not alter the viability of control cells, it reduced the death of IL-6-differentiated plasma cells by 60% in 24 h. Plasma cell apoptosis is therefore primarily caspase dependent.

The role of Fas in plasma cell apoptosis was then addressed. Flow cytometric analysis showed that Fas was highly expressed on control CESS cells but substantially reduced, although not eliminated, on IL-6-differentiated plasma cells (Fig. 1*B*). Confirming this observation, immunofluorescent staining revealed a prominent reduction of Fas on plasma cells expressing high levels of intracellular IgG (Fig. 1*C*). Moreover, FasL mRNA was undetectable in CESS cells before or after IL-6 differentiation by either RT-PCR or RNase protection assays (Figs. 1*D* and 2*A*). Thus, IL-6-differentiated plasma cells express reduced level of Fas and no FasL. The Fas pathway is unlikely to be instrumental in the death of these clonal plasma cells in vitro.

Expression of TRAIL, DR4 and DR5 on IL-6-differentiated human plasma cells

Apoptosis of IL-6-differentiated plasma cells in the absence of FasL prompted us to investigate the role of TRAIL in plasma cell death. RNase protection assays revealed that mRNAs encoding TRAIL, DR4, and DR5 were expressed in IL-6-differentiated plasma cells (IgG^{high}), at levels comparable with those observed in IL-6-refractory (IgG^{low}) and untreated CESS cells (Fig. 2*A*). The Fas mRNA levels were similarly maintained in IL-6-differentiated plasma cells (Fig. 2*A*), implying that reduction of Fas protein (Fig. 1) might occur at the posttranscriptional level. The maintenance of DR4 and DR5 expression was confirmed at the protein level by immunoblot analysis (Fig. 2*B*), where detection of DR5 but not DR4 in Jurkat T cells (15) served as a control for the Ab specificity. The purity of the plasma cell population was confirmed through the detection of high IgG levels, and tubulin expression controlled for protein loading (Fig. 2*B*). There was no evidence for the presence of DR3 or TNFR p55 mRNAs in plasma cells (Fig. 2*A*). The expression of TRAIL, DR4, and DR5 is therefore retained during IL-6 differentiation of human B lymphoblastoid cells to plasma cells. The reduction of surface Fas expression and the absence of FasL indicate that Fas is unlikely to be instrumental in the apoptosis of IL-6-differentiated plasma cells. The sustained expression of TRAIL and its receptors in plasma cells is consistent with a potential role for TRAIL in inducing plasma cell apoptosis in vitro.

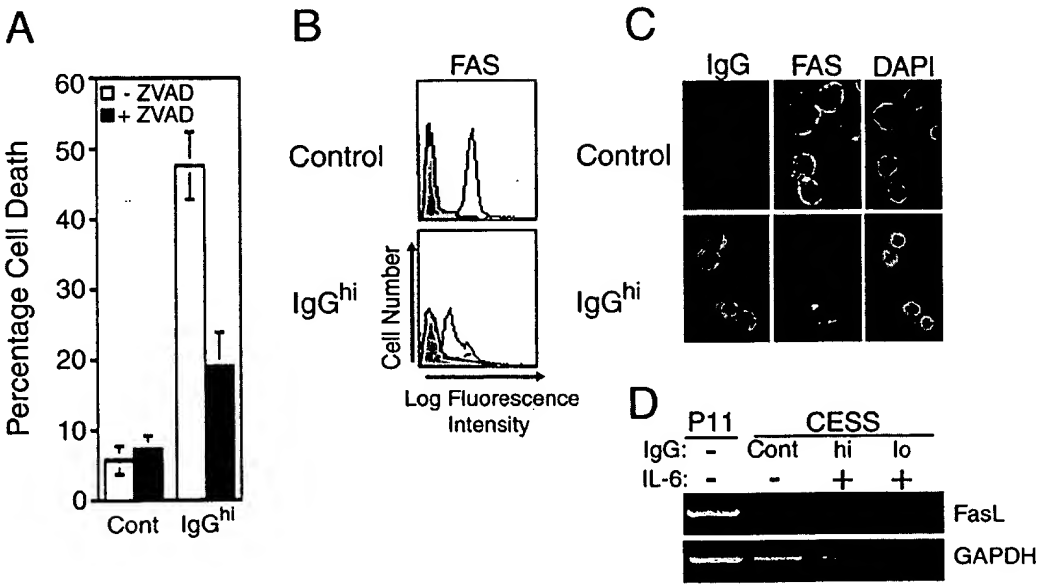

FIGURE 1. Reduction of Fas expression on plasma cells. *A*, Control lymphoblastoid CESS cells or IL-6-differentiated IgG^{high} plasma cells were incubated for 24 h in the absence (□) or presence (■) of ZVAD. The percent of cell death was determined by trypan blue staining; values represent three independent experiments. *B*, Flow cytometric analysis (open histogram) of surface Fas expression on control CESS cells or IL-6-differentiated plasma cells. The shaded areas represent signals obtained from an isotype control Ab. *C*, Immunofluorescence analysis of Fas (green) and intracellular IgG (red) expression in control CESS cells and IL-6-differentiated plasma cells. All cells were counterstained with 4',6'-diamidino-2-phenylindole (DAPI; blue) to detect nuclei. *D*, RT-PCR analysis of FasL mRNA expression in control (Cont), IL-6-differentiated (IgG^{high}), and IL-6-refractory (IgG^{low}) CESS cells and the positive control P11 cells. The analysis of GAPDH mRNA serves as a control for RNA loading.

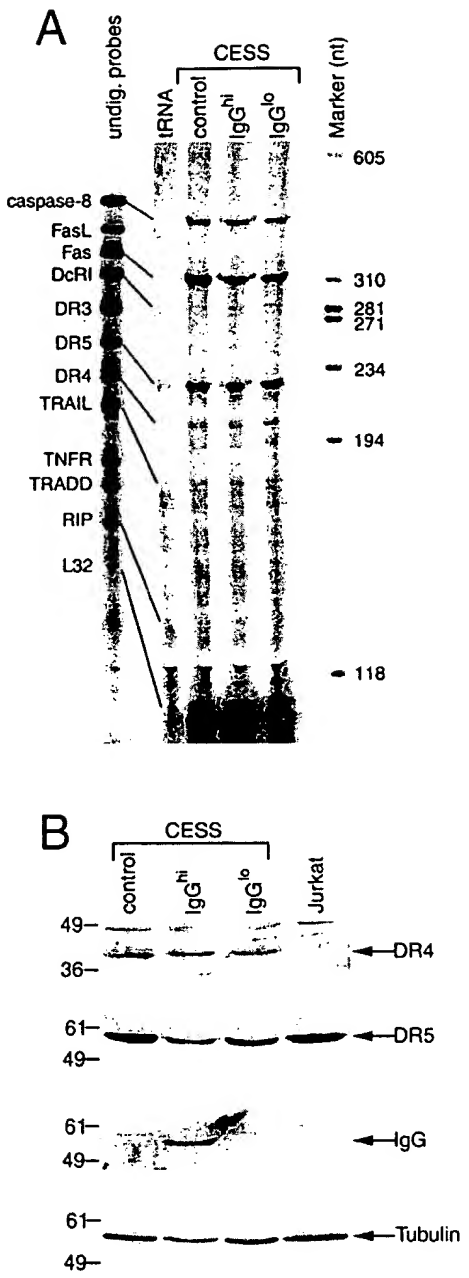

FIGURE 2. Expression of TRAIL ligand and receptors in human plasma cells. *A*, RNase protection assay of RNA isolated from untreated CESS cells (control), IL-6-differentiated plasma cells (IgG^{high}), and IL-6-refractory cells (IgG^{low}) with yeast tRNA as a negative control. The undigested (Undig.) riboprobes and their corresponding protected fragments are marked by individual lines. The migration of the molecular mass standard (nt) is indicated. TRADD, TNFR-associated death domain containing protein. *B*, Sequential immunoblot analysis of whole cell lysates for DR4, DR5, IgG, and tubulin expression prepared from control CESS cells, IL-6-differentiated plasma cells (IgG^{high}), IL-6-refractory cells (IgG^{low}), and Jurkat cells; 30 μg of total protein were resolved on a 10% SDS-PAGE gel. The molecular mass standards (kilodaltons) are indicated.

Endogenous and exogenous TRAIL induces apoptosis of IL-6-differentiated human plasma cells

The function of TRAIL in plasma cell apoptosis was characterized, first in IL-6-differentiated human IgG-secreting plasma cells due to the relative ease in enriching this plasma cell population. To determine whether endogenous TRAIL induces plasma cell death, IL-6-differentiated plasma cells were incubated with DR5-Fc, an inhibitory chimeric soluble TRAIL receptor comprised of the extracellular domain of DR5 fused to the Fc portion of human IgG (29) (Fig. 3). Indeed, DR5-Fc inhibited plasma cell death by 40% within 24 h, as assessed by trypan blue staining (Fig. 3, *top*). This correlated with a 4-fold increase in the relative amounts of IgG secreted into the medium during the 24-h period, as measured by ELISA (Fig. 3, *bottom*). Inhibition of cell death by DR5-Fc was specific to the DR5 extracellular domain, because the Fc portion of human IgG had no effect (Fig. 3). Thus, endogenous TRAIL functions to induce plasma cell death. The addition of soluble, trimerized recombinant human TRAIL further enhanced plasma cell death, whereas the presence of both DR5-Fc and TRAIL led to a marked reduction of plasma cell death and a corresponding increase in IgG secretion (Fig. 3). These results suggest that plasma cell death is induced by endogenous TRAIL and augmented by exogenous TRAIL.

Induction of cell death by TRAIL, however, did not extend to the control CESS lymphoblastoid cells (Fig. 3). They were intrinsically less apoptotic than IL-6-differentiated plasma cells, and were refractory to TRAIL-mediated killing at a concentration that enhanced plasma cell death (Fig. 3). TRAIL therefore preferentially induces the death of IL6-differentiated plasma cells.

TRAIL induces apoptosis of primary plasma cells generated in T-dependent immune responses

The susceptibility of IL-6-differentiated human plasma cells to TRAIL killing prompted us to address whether primary plasma cells are also direct targets of TRAIL-mediated apoptosis (Fig. 4). Primary mouse plasma cells were generated in vivo by immunization with NP-CGG, a T cell-dependent Ag and enriched to >70% homogeneity by selecting syndecan-1-positive cells from splenic B cells isolated on day 10 of immunization. As controls, resting and activated B cells present in the syndecan-1-negative

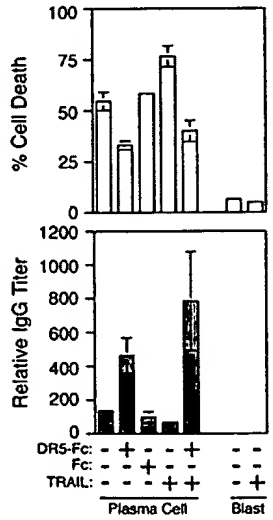

FIGURE 3. TRAIL-mediated apoptosis of human plasma cells. *Top*, Control CESS cells (blast) or IL-6-differentiated plasma cells were cultured for 24 h in the presence or absence of 1.0 μg/ml TRAIL, DR5-Fc, or the Fc portion of human IgG. Cell viability was determined by trypan blue exclusion, and the percent of plasma cell death was calculated by dividing the number of dead plasma cells by the total number of plasma cells in the culture. *Bottom*, ELISA of IgG secretion in the corresponding culture medium. Data represent the relative IgG titer required to obtain a single OD value that lies within the linear range. Data represent three independent cell populations from one experiment. Three independent experiments were performed with similar results.

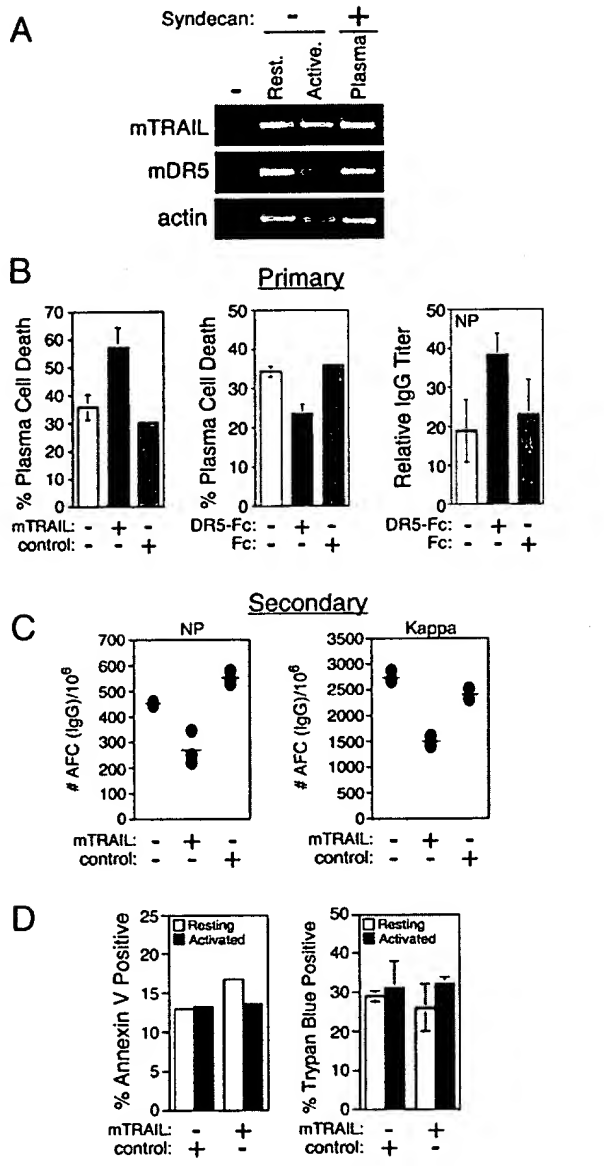

FIGURE 4. TRAIL induces apoptosis of primary plasma cells generated in a T-dependent immune response. *A*, RT-PCR analysis of the levels of murine TRAIL and DR5 mRNA, and the control actin mRNA present in resting and activated B cells, and syndecan-1-positive plasma cells isolated on day 10 of a primary NP-CGG response. As a negative control, the RT-PCR were performed in the absence of template (−). *B*, The in vivo-generated, syndecan-1-positive plasma cells were cultured alone (□) or with TRAIL-expressing 2PK3 cells (mTRAIL, ■) or the parental 2PK3 (control, ▨) cells at a 10:1 ratio for 5 h before determination of cell death by trypan blue staining (*left panel*). In vivo-generated plasma cells were plated onto CD40L-expressing L cells at a 10:1 ratio in the absence (□) or presence (■) of DR5-Fc or Fc (▨) (1 μg/ml). Cell viability was determined by trypan blue exclusion after a 5-h incubation (*middle panel*), and the relative amount of NP-specific IgG secreted into the medium for a total of 20 h was determined by ELISA (*right panel*). *C*, ELISPOT analysis of NP-specific (*left panel*) and κ-specific (*right panel*) IgG-secreting cells (Ab-forming cells; AFC) generated in a secondary NP-CGG immune response after a 4-h incubation ex vivo on mTRAIL or control 2PK3 cells as indicated. *D*, Resting (□) and activated (■) B cells were cultured on mTRAIL or parental 2PK3 (control) cells at a 10:1 ratio. The percentage of annexin V^+ propidium iodide $(PI)^-$ cells (*left panel*) and trypan blue-positive (*right panel*) cells were determined after 24 h in culture. For each of the above conditions, the values represent independent analysis of cells isolated from three mice.

fraction were subsequently separated by Percoll gradient centrifugation (30). TRAIL and DR5 (there is no DR4 in mice) mRNAs were expressed at comparable levels in primary resting, activated, and plasma cells, as indicated by RT-PCR analysis (Fig. 4*A*).

Having verified the expression of TRAIL ligand and receptor in primary plasma cells, we then determined whether they were subject to TRAIL killing, by incubation with 2PK3 cells stably expressing the trimerized mTRAIL or the parental cell line as a negative control (31). Plasma cell death was enhanced 2-fold within 5 h of coincubation with mTRAIL ($p = 0.03$), but not with the control cells (Fig. 4*B*, *left panel*). Conversely, it was reduced by 30% by incubation with DR5-Fc ($p = 0.02$), but not with Fc (Fig. 4*B*, *middle panel*). The reduction of plasma cell death by DR5-Fc was corroborated by a 2-fold increase ($p = 0.03$) in the amount of NP-specific IgG Ab secreted in the medium (Fig. 4*B*, *right panel*). Thus, endogenous and exogenous TRAIL cooperate to induce the death of primary plasma cells generated in a primary T cell-dependent response.

To further confirm that TRAIL induces apoptosis of primary plasma cells generated in vivo, the loss of plasma cells secreting NP-specific IgG in response to TRAIL was assayed functionally by ELISPOT (Fig. 4*C*). NP-specific plasma cells were first amplified in vivo in a secondary response elicited 5 wk after primary NP-CGG immunization. Splenic B cells were isolated at the peak of the secondary Ab response (day 6) and directly cultured, without selection for syndecan-1-positive cells, for 4 h in medium, alone or together with mTRAIL or the control 2PK3 cells (Fig. 4*C*). mTRAIL, but not the control cells, induced a 2-fold reduction of the number of NP-specific IgG plasma cells (Ab-forming cell) ($p = 0.006$) as well as the polyclonal IgGκ-secreting plasma cells ($p = 0.03$). Thus, TRAIL directly induces the death of Ag-specific and polyclonal, class-switched, primary plasma cells generated in a T-dependent immune response.

Incubation with mTRAIL-expressing cells ex vivo, however, did not enhance apoptosis of resting or activated splenic B cells isolated from a primary NP response, as determined by either the annexin V-binding activity of early apoptotic cells or trypan blue staining of dead cells (Fig. 4*D*). Primary resting and activated mouse B cells are therefore refractory to TRAIL-mediated killing ex vivo, in agreement with our observation during IL-6 differentiation of human lymphoblastoid cells (Fig. 3). TRAIL therefore preferentially induces the death of primary plasma cells but not resting or activated B cells in a T-dependent immune response.

TRAIL-mediated apoptosis of IL-6-differentiated human plasma cells is primarily caspase dependent

TRAIL has been shown to initiate the caspase cascade by recruitment and activation of caspase-8 (15–17). Spontaneous and TRAIL-induced death of IL-6-differentiated plasma cells are inhibited by ZVAD (Figs. 1*A* and 5*A*), suggesting that the TRAIL death signals are mediated by caspases. Consistent with this possibility, both isoforms of procaspase-8 (55 and 53 kDa) as well as procaspases-3 and -7 were abundantly expressed in IL-6-differentiated IgG plasma cells as determined by immunoblotting (Fig. 5*B*). The initiator caspase-8 was activated within 1 h of TRAIL stimulation, as indicated by the emergence of its cleavage products. This was rapidly followed by the activation of the effector caspases-7 and -3, also in agreement with observations in other cell types (15–17, 34). Although the cleavage of all three caspases in response to TRAIL was inefficient in IL-6-differentiated plasma cells (Fig. 5*B*), the levels of cleavage were comparable with those observed by others in a subset of cell lines including human B cell lines and colon and lung carcinomas (16, 17). Moreover, activation of these three caspases was also very modest after prolonged (7 h)

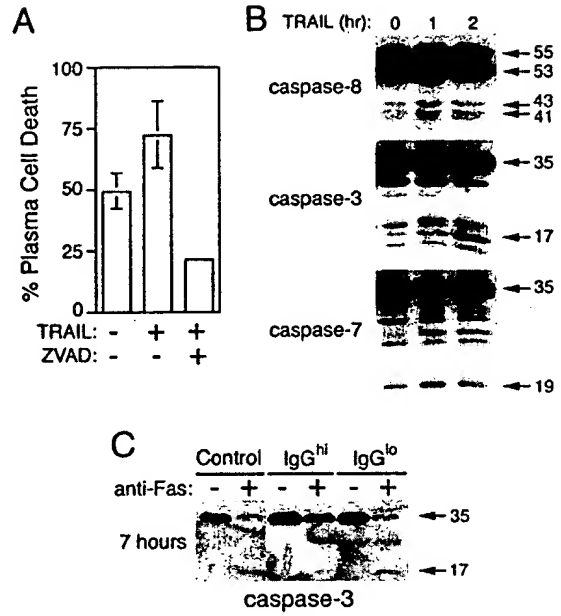

FIGURE 5. TRAIL-mediated plasma cell death is primarily caspase dependent. *A*, Inhibition of TRAIL-mediated apoptosis of IL-6-differentiated plasma cells by ZVAD. Cells were coincubated with or without 0.1 μg/ml FLAG-tagged TRAIL, together with 1 μg/ml of the FLAG enhancer Ab, and 20 μM ZVAD for 24 h, and viability was determined by trypan blue exclusion. *B*, Immunoblot analysis of cleavage of caspases-8, -3, and -7 in IL-6-differentiated plasma cells treated with or without 0.1 μg/ml FLAG-tagged TRAIL together with 1 μg/ml concentrations of the FLAG enhancer Ab for the hours indicated. *C*, Immunoblot analysis of caspase-3 cleavage in control CESS cell, IL-6 differentiated plasma cells (Ig^{high}) and refractory cells (Ig^{low}) treated with or without an anti-Fas Ab for 7 h as indicated. The molecular mass standards (kilodaltons) are shown.

Fas cross-linking, in striking contrast to their efficient activation in the control lymphoblastoid cells under the same Fas cross-linking conditions (Fig. 5*C* and data not shown). Activation of caspases-8, -3, and -7 is therefore inefficient in plasma cells by either TRAIL or Fas.

The reduction of TRAIL-induced death by ZVAD and the activation of the caspase machinery in response to TRAIL demonstrate that TRAIL-mediated plasma cell apoptosis in vitro is primarily caspase dependent (Fig. 5). However, protection from apoptosis by ZVAD was incomplete, implying that a caspase-independent mechanism may function in concert with the caspase-dependent pathway to induce plasma cell apoptosis in response to TRAIL.

Decreased CD40 expression and inactivation of NF-κB in IL-6-differentiated human plasma cells

Our data point to preferential killing of plasma cells, but not B cells before terminal differentiation, by TRAIL despite comparable levels of expression of both the ligand and receptors. This raises the possibility that either functional TRAIL/DR complexes cannot form on activated (lymphoblastoid) cells, or that plasma cells have specifically lost the ability to respond to one or more survival signals. To address the latter possibility, we investigated the regulation of the TRAF/NF-κB survival pathway, which is constitutive in EBV-transformed lymphoblastoid cells (26, 27) but may no longer operate in IL-6-differentiated plasma cells (22) due to loss of LMP1 expression.

Electrophoretic mobility shift assays showed that NF-κB was highly activated in control and IL-6-refractory CESS cells, as evidenced by the formation of NF-κB-DNA complexes consisting of p50/p65, p50/RelB, and p50/p50 (Fig. 6*A*, *lanes 1* and *2*). However, the NF-κB DNA-binding activity was drastically reduced in IL-6-differentiated plasma cells (Fig. 6*A*, *lane 3*). Although TRAIL stimulation can activate NF-κB in other cells (35–38), it did not enhance the NF-κB DNA-binding activity in either IL-6-differentiated plasma cells or refractory cells (Fig. 6*A*, *lanes 4* and *5*). The loss of NF-κB activity could not be attributed to an overall reduction in DNA-binding activity of cellular transcription factors, because the Oct-1 DNA binding activity was comparable in each lysate (Fig. 6*B*). Moreover, coordinated with the loss of LMP1 expression (22), the expression of CD40, a potent activator of NF-κB in B lineage cells (5, 6), was also profoundly reduced in IL-6-differentiated plasma cells (Fig. 6*C*). This correlated with the failure of CD40L to delay or rescue apoptosis of these plasma cells (data not shown). Thus, terminal differentiation of lymphoblastoid cells by IL-6 correlated with coordinated loss of CD40 expression and NF-κB activation.

Reduction of CD40 and Fas expression in primary mouse plasma cells

Spontaneous apoptosis ex vivo of primary plasma cells generated in the NP response also could not be delayed or prevented by stimulation with CD40L (Fig. 4*B*), suggesting that the expression of CD40 might be similarly reduced in primary plasma cells. Indeed, the expression of CD40 on primary plasma cells (Syn^{high}/

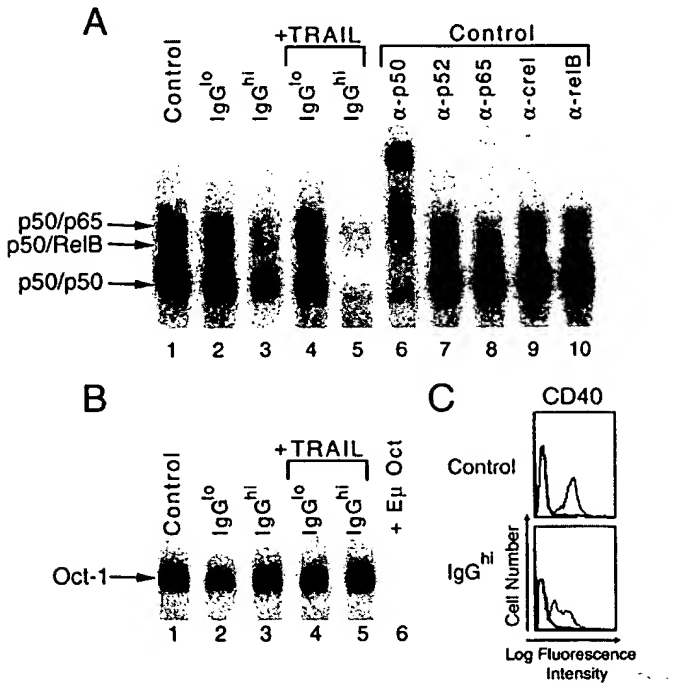

FIGURE 6. Loss of NF-κB DNA binding activity and CD40 expression in human plasma cells. *A*, EMSA of the NF-κB DNA-binding activity was performed on lysates prepared from control CESS cells (*lane 1*), IL-6-refractory cells (IgG^{low}) (*lanes 2* and *4*), and IL-6-differentiated plasma cells (IgG^{high}) (*lanes 3* and *5*) cultured in the absence or presence of 0.5 μg/ml TRAIL as indicated. The various NF-κB subunits in the DNA-protein complexes was identified by specific Abs (*lanes 6–10*). *B*, EMSA of the Oct-1 DNA-binding activity was performed on the same lysates shown in *A* without competition (*lanes 1–5*). Competition experiments were performed with an unlabeled oligonucleotide containing the octamer site from the Ig Eμ Oct enhancer and the control CESS cell lysate (*lane 6*). *C*, FACS analysis of CD40 expression (open area) on control CESS cells and IL-6-differentiated plasma cells (Ig^{high}).

$B220^{low}$) was significantly lower than that on B cells (Syn^{low}/$B220^{high}$) from the same immune response (Fig. 7*A*). To address the possibility that the expression of CD40 might be temporally regulated during B cell terminal differentiation, primary mouse plasma cells were generated in vitro by sequential coculture of resting splenic B cells with CD40L-expressing L cells and then MC3T3 osteoblastic cells in the presence of IL-6. Under these conditions, the majority of B cells (85%) entered the cell cycle by day 3 based on 5-bromo-2′-deoxyuridine uptake and continued to proliferate until around day 7, when cell cycle arrest began. From day 9 onward, coculture with MC3T3 cells facilitated terminal differentiation, leading to plasma cells that had lost B220 and MHC class II, expressed syndecan-1 and secreted IgM (W. Zhang and S. Chen-Kiang, unpublished observations). The expression of CD40 was maintained on activated B cells (day 4), reduced as cells withdrew from the cell cycle (day 7), and further decreased in plasma cells (day 11) to levels substantially below that of resting B cells (Fig. 7*B*). Together, these in vivo and in vitro results demonstrate that CD40 expression is progressively reduced during terminal differentiation of primary B cells.

The reduction of CD40 expression suggests that the NF-κB survival pathway may be coordinately inactivated in primary plasma cells. To address this possibility, we determined the expression of A1 in activated B cells and plasma cells as a functional readout for NF-κB activity because A1 is a target for both NF-κB and CD40 (39, 40). The A1 protein level was markedly lower in plasma cells (Syn^+) compared with activated B cells (Syn^-) generated in the primary NP response, whereas Bcl-2 and tubulin levels remained unchanged (Fig. 7*C*). These results confirmed that CD40 and a NF-κB target gene, *A1*, are selectively and coordinately reduced in primary plasma cells.

Surface Fas expression was prominently elevated on CD40L-activated B cells (Fig. 7*B*), in agreement with previous reports (41). Of interest, Fas expression was also drastically reduced on plasma cells (day 11), to levels characteristic of resting B cells (Fig. 7*B*). Fas and CD40 are therefore coordinately regulated during terminal differentiation of primary B cells initiated by CD40 signaling. Together with the reduction of Fas on IL-6-differentiated human plasma cells (Fig. 1*B*), the loss of surface Fas expression appears to be common in plasma cells.

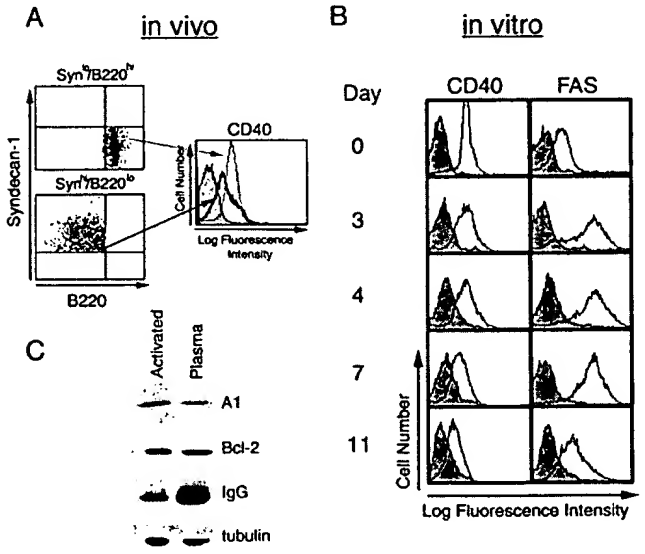

FIGURE 7. Decreased CD40, Fas, and *A1* expression in primary plasma cells. *A*, FACS analysis of CD40 expressed on in vivo-generated Syn^{high}/$B220^{low}$ plasma cells (black line) and Syn^{low}/$B220^{high}$ activated B cells (gray line). The shaded area represents unstained cells. *B*, FACS analysis of CD40 and Fas expression (open areas) during in vitro terminal differentiation of primary resting splenic mouse resting B cells with CD40L and IL-6 as described in *Materials and Methods*. The shaded areas represent the fluorescence obtained with an isotype control Ab. *C*, Immunoblot analysis of the expression of *A1*, Bcl-2, Ig, and tubulin in 10 μg of whole cell lysate prepared from activated and plasma cells generated in the primary NP-CGG response.

Discussion

In this study, we demonstrate that primary plasma cells are susceptible to killing mediated by endogenous and exogenous TRAIL and that TRAIL-mediated apoptosis does not extend to resting or activated primary B cells despite comparable levels of ligand and death receptor expression. This sensitivity of plasma cells to TRAIL-mediated apoptosis may relate to reduced CD40 expression and NF-κB survival signals.

Induction of plasma cell apoptosis by TRAIL

The death of plasma cells is tightly coordinated with cell cycle arrest and cellular differentiation to ensure their rapid elimination at the end of a humoral immune response (2, 3, 22, 23). Here, we provide the first direct evidence that TRAIL mediates spontaneous and accelerated plasma cell death in two independent model systems: primary plasma cells generated in a T-dependent immune response; and human IgG plasma cells differentiated in vitro by IL-6. First, we showed that the expression of TRAIL and the DR death receptors were maintained in Ag-specific and polyclonal mouse plasma cells generated in the 4-hydroxy-3-nitrophenyl (NP) response (Fig. 4*A*) as well as IL-6-differentiated human plasma cells (Fig. 2). Next, we demonstrated that both plasma cells were susceptible to killing mediated by endogenous and exogenous TRAIL ex vivo (Figs. 3 and 4). In light of the lack of understanding of TRAIL function in primary cells, lymphocytes in particular, these findings have significant implications for the mechanisms that control primary plasma cell apoptosis.

The susceptibility of plasma cells to TRAIL-mediated killing is in part determined by temporal changes in the composition of death receptors during B cell terminal differentiation. Contrasting the sustained expression of DR, Fas protein expression was drastically reduced in IL-6-differentiated human plasma cells, and in primary mouse plasma cells (Figs. 1 and 7*B*). FasL expression was absent in the former (Figs. 1*D* and 2*A*). Although cross-linking of Fas can be facilitated by an extracellular domain in the absence of ligand (42), the low level of Fas expressed on plasma cells would likely preclude efficient ligand-independent Fas oligomerization. Fas signaling is therefore not responsible for the rapid apoptosis of IL-6-differentiated plasma cells in vitro (Figs. 3 and 4) and unlikely to be the primary mechanism that controls the death of primary plasma cells.

The death of plasma cells, either spontaneous or induced by exogenous TRAIL, is caspase dependent based on its inhibition by ZVAD (Fig. 5). The extent to which TRAIL activates the caspase machinery has been shown to vary greatly according to cell types (15–17). In some cell lines, including B cell lines, the levels of caspase cleavage (15–17) are comparable with our findings in plasma cells (Fig. 5). Despite the inefficiency of caspase cleavage by TRAIL, which may be inherent to specific cell types, the ability of ZVAD to block the majority of TRAIL-induced killing suggests that plasma cells respond to TRAIL death signals primarily through caspase-dependent mechanisms. However, this does not preclude the involvement of a caspase-independent pathway that may function in concert with activation of the caspase-8 pathway to promote plasma cell death. This possibility is consistent with the

finding that TRAIL can also activate a caspase-independent pathway through the RIP serine/threonine kinase to promote cell death (18). In addition, plasma death is unlikely to be induced exclusively by TRAIL, because neutralization of TRAIL killing by DR5-Fc significantly reduced, but did not block, plasma cell death (Figs. 3 and 4). Further studies are required to determine the relative contributions of TRAIL and additional death signals to plasma cell death and the intracellular pathways that mediate TRAIL-mediated plasma cell death.

TRAIL-mediated apoptosis is coincident with inactivation of the CD40-NF-kB signaling pathway

TRAIL induces apoptosis of terminally differentiated plasma cells, but not resting or activated primary mouse B cells or human lymphoblastoid cells (Figs. 3 and 4). Because the expression of key components of the TRAIL death pathway (TRAIL, DR4, and DR5) does not vary during B cell terminal differentiation (Figs. 2 and 4), this differential sensitivity of TRAIL-mediated killing must be determined by other factors. The most likely possibilities are regulated assembly of the TRAIL death-inducing signaling complex and altered balance between intracellular survival and apoptotic signals.

Inactivation of the CD40-NF-κB pathway during B cell terminal differentiation may contribute to the selective killing of plasma cells by TRAIL (Figs. 6 and 7). Although CD40 expression is believed to be constitutive in mature B cells, our results revealed that it is in fact markedly reduced in primary mouse plasma cells generated in vivo and in vitro as well as IL-6 differentiated human plasma cells (Figs. 6 and 7). This correlates with the failure of CD40L to protect plasma cells from apoptosis mediated by endogenous and exogenous TRAIL (Fig. 4*B*; J. Ursini-Siegel, W. Zhang and S. Chen-Kiang, unpublished observations). In the case of human lymphoblastoid cells, the extinction of LMP1 expression during terminal differentiation by IL-6 further ensures that the NF-κB pathway no longer functions in plasma cells (22). Confirming this prediction, the p50/p50, p50/RelB, and p50/p65 NF-κB DNA-binding activities were drastically reduced in IL-6-differentiated plasma cells and could not be restored by TRAIL (Fig. 6).

p50 and p65 are essential for the survival of primary lymphocytes (43, 44) and highly activated in freshly isolated primary resting and activated B cells (30). NF-κB may attenuate TRAIL-mediated killing through activation of specific Bcl-2 family proteins, based on the inverse correlation between sensitivity to TRAIL killing and expression of a specific NF-κB target gene *A1* in primary plasma cells and activated B cells (Fig. 7). In addition to modulating the intracellular balance between survival and death signals, NF-κB may also impair the assembly and signaling of an active TRAIL/death-inducing signaling complex through induction of DcR1 and c-FLIP expression (45–47). It would be of interest to determine the expression of DcR1 and c-FLIP in plasma cells and their roles in TRAIL-mediated apoptosis.

In the context of a humoral immune response, CD40 has dual functions in B cell activation and survival, and NF-κB is essential for the transcription of Ig genes (48). Moreover, CD40 engagement of germinal center B cells induces memory B cell formation but inhibits their terminal differentiation into Ab-secreting plasma cells (1, 49, 50), and removal of CD40 signals allowed germinal center B cells to undergo proper terminal differentiation (49). Thus, reduction of CD40 expression and inactivation of NF-κB during B cell terminal differentiation must be exquisitely regulated. Consistent with this possibility, we show that reduction of CD40 expression on activated B cells is coincidental with the onset of cell cycle arrest and continues progressively during subsequent differentiation to plasma cells in vitro (Fig. 7). On this basis, we suggest that the reduction of CD40 expression promotes not only B cell terminal differentiation but also the decline of CD40-activated NF-κB activity, at a point when plasma cells have accumulated and secreted sufficient amounts of Ig, may in fact signal the end of their life span.

Conditional induction of apoptosis of tumor and primary cells by TRAIL

TRAIL is thought to preferentially kill transformed cells of various lineages in vitro, including B and T cell lymphomas and multiple myeloma cells (7, 8, 10–12). Induction of apoptosis by the administration of TRAIL in vivo has been shown to reduce tumor incidence in experimental mouse models of colon carcinoma (9) and mammary adenocarcinoma (10). Conversely, neutralization of endogenous TRAIL signals led to accelerated liver metastases (51). The protection of HL60 cells from TRAIL-mediated killing by p65 activation (52) and sensitization of myeloma cells to TRAIL killing by inactivation of NF-κB (13, 53), however, suggest that susceptibility of tumor cells to TRAIL-mediated apoptosis may also be determined by the balance between apoptosis and survival signals mediated by NF-κB.

With the exception of human astrocytes and prostate epithelial cells, most human primary cells are highly resistant to TRAIL-mediated apoptosis (9, 10, 54). However, they can be sensitized to TRAIL-mediated killing by CD3 ligation in the case of primary human thymocytes (55) and by inhibition of protein synthesis of primary human thyroid follicular cells and keratinocytes (56, 57). Our finding that primary resting and activated B cells are refractory to TRAIL killing does not preclude the possibility that additional signals may render them sensitive to TRAIL killing. Elucidating the pathways that mediate and modulate TRAIL-induced apoptosis in primary B lineage cells should help to better understand the control of TRAIL-mediated apoptosis of both normal and malignant plasma cells.

Acknowledgments

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TRAIL receptor-2 signals apoptosis through FADD and caspase-8

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Certain cytokines of the tumour-necrosis factor (TNF) family and their cognate receptors (collectively named death receptors) are potent inducers of programmed cell death (apoptosis)[1]. One such protein is the cell-surface receptor Fas, which, upon ligand binding, trimerizes and recruits the adaptor protein FADD through the cytoplasmic death domain of Fas. FADD then binds and activates procaspase-8 (ref. 1). TRAIL, the most recently identified member of the TNF family of death ligands, can induce apoptosis in a wide variety of tumour cells but not in normal cells[2]. TRAIL induces apoptosis through two death-domain-containing receptors, TRAIL-R1 (also called death receptor (DR) 4)[3] and TRAIL-R2 (or DR5)[4–9]. Investigation of the intracellular signalling pathways responsible for TRAIL-receptor-induced apoptosis has produced controversial results. Genetic evidence[10,11] indicates the

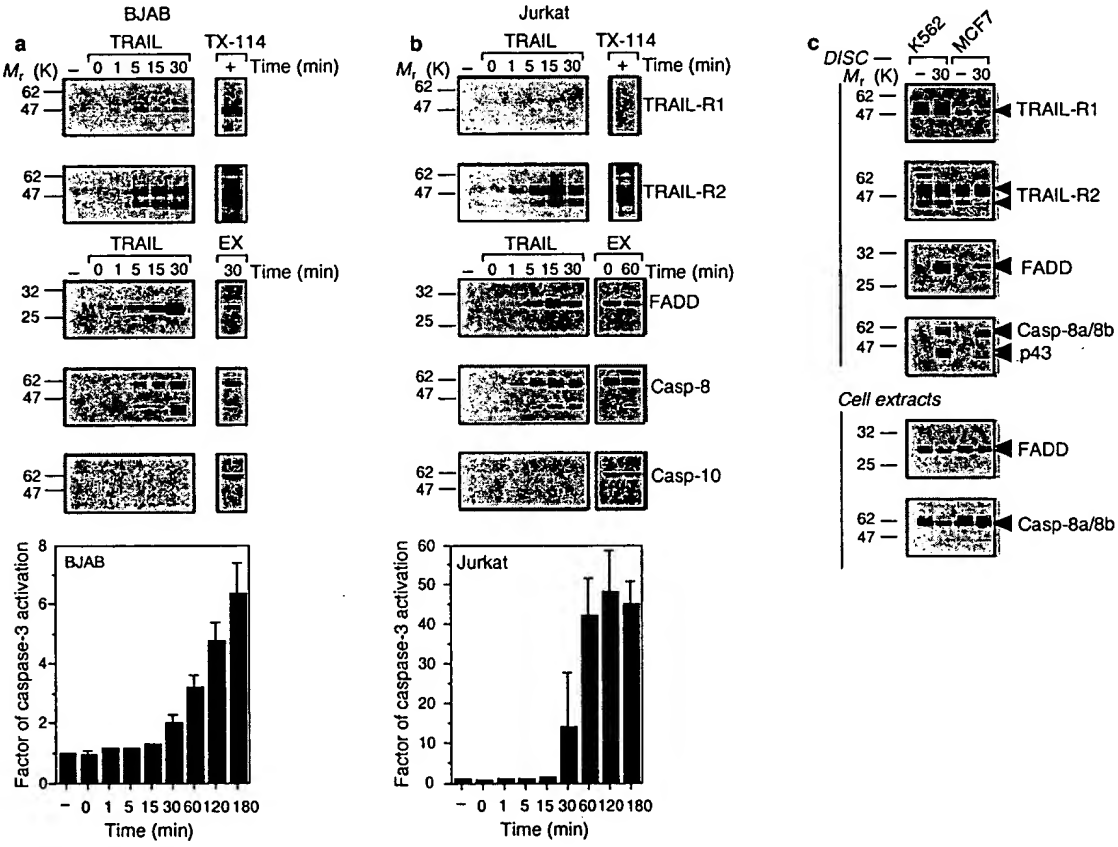

Figure 1 Time course of recruitment of FADD and caspase-8 to TRAIL receptors. **a**, BJAB Burkitt lymphoma cells expressing TRAIL-R1 and TRAIL-R2 were treated with crosslinked recombinant Flag-tagged soluble TRAIL (sTRAIL) for the indicated time periods and then lysed (see Methods). Upper panels, the assembled death-inducing signalling complexes (DISCs) were immunoprecipitated with protein A and analysed by western blotting using antibodies to TRAIL-R1, TRAIL-R2, FADD, caspase-8 and caspase-10 (the latter was detected using either a polyclonal or a monoclonal (data not shown) anti-caspase-10 antibody). In unstimulated controls (–), sTRAIL and anti-Flag M2 monoclonal antibody were added after lysis to immunoprecipitate non-stimulated TRAIL receptors. We detected two distinct TRAIL-R2 species, which probably correspond to the two alternatively spliced forms described[7]. Cell extracts (EX) or Triton X-114 extracts (TX114) were analysed for the presence of receptors before TRAIL addition (these results are denoted by '+'), and for FADD and caspases 30 min after TRAIL addition. Lower panel, the kinetics of caspase-3 activation was determined using the synthetic substrate DEVD–AMC, which fluoresces after it is cleaved. **b**, Corresponding analysis of Jurkat T cells, which express TRAIL-R2 but not TRAIL-R1. **c**, TRAIL-R DISC formation was analysed in K562 chronic myelogenous leukaemia cells and MCF-7 breast adenocarcinoma cells before (–) and 30 min after (30) the addition of crosslinked sTRAIL. TRAIL receptors (but not FADD or caspase-8) are immunoprecipitated even when sTRAIL is added after cell lysis.

possible involvement of a FADD-like molecule and caspase-10 rather than of FADD itself and caspase-8. Here we characterize the signalling complex of TRAIL-R2 that is assembled in response to ligand binding. We provide evidence that FADD and caspase-8, but not caspase-10, are recruited to the receptor. Moreover, mutant cell lines that lack FADD or caspase-8 are resistant to TRAIL-induced death. Thus, TRAIL-R2 and Fas death signals rely on identical signalling molecules.

The Fas signalling pathway was elucidated by means of analysis of its death-inducing signalling complex (DISC)[12], that is, by studying the endogenous (and not overexpressed) proteins that are recruited to activated Fas. We therefore took a similar approach with respect to TRAIL signalling, using recombinant Flag-tagged soluble TRAIL (sTRAIL) and anti-Flag-antibodies to immunoprecipitate the TRAIL-receptor DISC from BJAB cells. Flag-tagged sTRAIL does not induce cell death unless it is crosslinked with anti-Flag antibodies, thereby mimicking membrane-bound TRAIL[13]. At various time points after addition of the crosslinked ligand, the TRAIL-receptor DISC was immunoprecipitated and assayed for proteins known to be involved in Fas signalling. BJAB cells express TRAIL-R1 and TRAIL-R2 complementary DNA[14], and both TRAIL-receptor proteins were incorporated into the DISC, along with FADD and caspase-8, after 5 min of stimulation (Fig. 1). Maximal recruitment of all proteins was observed after 30 min, at which time caspase-8 was converted from its precursor into the active processed form, as shown by the appearance of the p43 fragment[12] in the DISC. In contrast, the substantial amounts of caspase-10 detected in the cytoplasm were neither processed nor recruited to the DISC. Activation of caspase-8 was rapidly followed by the appearance of caspase-3 activity in the cytoplasm (Fig. 1a).

As the TRAIL-receptor DISC isolated from BJAB cells contains a mixture of TRAIL-R1 and TRAIL-R2, we next analysed Jurkat T cells, which have been shown previously to express TRAIL-R2, but not TRAIL-R1, cDNA[14]. In agreement with this, we found that only TRAIL-R2 bound to immunoprecipitated TRAIL in Jurkat cells (Fig. 1b). Recruitment of FADD and caspase-8 to activated TRAIL-R2 was slightly faster than in BJAB cells — binding was already detected 1 min after ligand addition. However, the more rapid association and activation of caspase-8 was not reflected in an earlier appearance of caspase-3 activity (Fig. 1b). As in BJAB cells, recruitment of cytoplasmic caspase-10 to TRAIL-R2 was not detected, and, in contrast to caspase-8, no processing of caspase-10 was observed in the cytoplasm 60 min after TRAIL addition (Fig. 1b) or at any later time point (data not shown). To exclude the possibility that the incorporation of FADD and caspase-8 is restricted to cells of lymphocytic origin only, we studied DISC assembly in K562 chronic myelogenous leukaemia cells and MCF-7 breast adenocarcinoma cells (Fig. 1c). Again, we found that both caspase-8 and FADD, but not caspase-10 (data not shown), were constituents of the TRAIL-R2 DISC.

The finding that FADD and caspase-8 are recruited to the TRAIL-R2 (and possibly TRAIL-R1) DISC does not necessarily mean that these signalling components are essential for the transmission of signals leading to cell death. We therefore studied TRAIL-induced apoptosis in mutant Jurkat T cells that lack either FADD or caspase-8 (Fig. 2). Both cell lines are resistant to Fas-induced death signals[15]. Incorporation of caspase-8 into the TRAIL-receptor DISC was defective not only in cells lacking caspase-8 but also in FADD-deficient cells, indicating, first, that for caspase-8 recruitment, FADD cannot be substituted by a FADD-like protein, and second, that caspase-10 cannot replace caspase-8 (Fig. 2a). Consequently, FADD-deficient Jurkat cells were still viable at extremely high TRAIL concentrations (1 μg ml^{-1}) (Fig. 2b), in contrast to wild-type Jurkat cells which underwent extensive apoptosis. Caspase-8-deficient Jurkat cells were also completely resistant to TRAIL, indicating that FADD and caspase-8 are both essential and non-redundant molecules in the TRAIL-R2 signalling pathway, at least in the cell types studied.

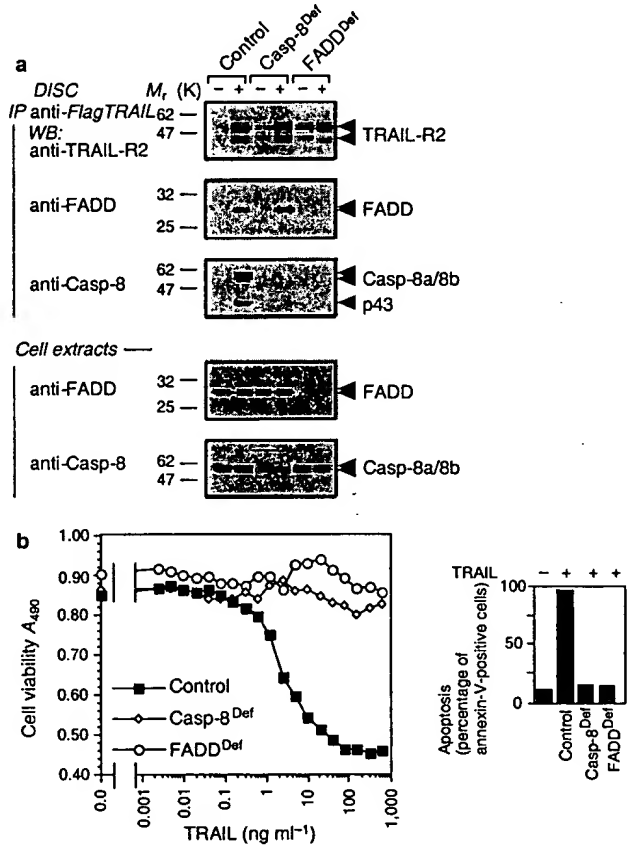

Figure 2 Impaired TRAIL-R2 signalling pathway in mutant Jurkat T-cell lines that are defective in caspase-8 or FADD expression. **a**, Analysis of TRAIL-receptor death-inducing signalling complexes (DISCs) in wild-type (control), caspase-8-deficient (Casp-8^{Def}) or FADD-deficient (FADD^{Def}) Jurkat T cells. DISCs were immunoprecipitated before (–) or 30 min after (+) the addition of TRAIL and analysed as described in Fig. 1. **b**, Wild-type and mutant Jurkat cells were incubated with increasing concentrations of crosslinked recombinant Flag-tagged soluble TRAIL (sTRAIL), and cell survival (left panel) and apoptosis (right panel; per cent annexin-V-positive cells; 100 ng ml^{-1} crosslinked sTRAIL) were determined after 16 h.

Our results agree with studies that showed the interaction of FADD with overexpressed TRAIL-R1 and TRAIL-R2 and the inhibition of TRAIL-induced cell death by dominant-negative mutant FADD[14,16]. However, studies that have reached opposite conclusions[3,4,6] remain unexplained. In particular, overexpression of TRAIL-R1 has been found to induce apoptosis in FADD-deficient embryonic fibroblasts[10], indicating that a FADD-like molecule may be able to transduce the death signal of TRAIL-R1. This possibility cannot be excluded from our study, as it is limited to the assembly of the TRAIL-R2 DISC.

Although our analysis of the TRAIL-R2 DISC provides support for the importance of caspase-8 in TRAIL death signals, it failed to identify a similar function for caspase-10, which was suggested by overexpression studies[4,11] and by the finding that dendritic cells from individuals expressing a mutant caspase-10 are resistant to TRAIL signalling[11]. TRAIL signalling pathways may therefore differ in cells from different origins. Alternatively, a catalytically inactive caspase-10 mutant may act as a non-releasable substrate trap for caspase-8, thereby inactivating the caspase, as has been proposed for FLIP[17] (with which caspase-10 shares overall structural homology). This would also explain the unexpected finding that mutant caspase-10 blocks Fas signalling[11]. We conclude, having studied the

assembly of the TRAIL-receptor DISC under physiological conditions, that the TRAIL-R2 and Fas pro-apoptotic signalling pathways have been conserved during evolution, as both require FADD and caspase-8. □

Methods

Cell lines.

The generation and analysis of FADD-deficient, caspase-8-deficient and control Jurkat T-cell clones have been described previously[15,16]. Jurkat T cells, BJAB Burkitt lymphoma cells, K 562 chronic myelogenous leukaemia cells and MCF-7 breast adenocarcinoma cells were maintained in RPMI-1640 (Life Sciences, Basel, Switzerland) supplemented with 10% FCS and antibiotics.

Antibodies

Rabbit polyclonal anti-TRAIL-R1 (antibody DR4CT), anti-TRAIL-R2 (antibody DR5ID) and anti-caspase-10 antibodies were from Alexis. Mouse anti-human-caspase-8 monoclonal antibody was from MBL (Nagoya, Japan); mouse anti-human-caspase-10 monoclonal antibody was from R&D systems; mouse anti-human-FADD monoclonal antibody was from Transduction Laboratories; and mouse anti-Flag monoclonal antibody M2 was from Sigma.

DISC analysis.

Cells were grown to densities between 1 and 2×10^6 cells ml^{-1} in 2-l roller bottles. MCF-7 cells were grown in 15-cm plates and collected in PBS supplemented with 2 mM EDTA. 10^8 cells (per condition) were collected by centrifugation at 400*g* (for MCF-7 cells we used ~4×10^7 cells per condition). The resulting cell pellet was resuspended in 2 ml prewarmed complete RPMI (5×10^7 cells ml^{-1}) and the tube was transferred into a water bath at 37 °C. Recombinant human soluble Flag-tagged TRAIL (sTRAIL; Alexis; 20 μg ml^{-1}) and anti-Flag monoclonal antibody M2 (60 μg ml^{-1}) were premixed for 15 min on ice. Cells were stimulated in a final volume of 2 ml with 500 ng ml^{-1} sTRAIL and 1.5 μg ml^{-1} M2. In unstimulated controls, 1 μg sTRAIL and 1 μg M2 were added after lysis to immunoprecipitate non-stimulated TRAIL receptors. Cell suspensions were incubated for the indicated time periods at 37 °C and the reaction was stopped by the addition of 10 ml ice-cold PBS. The cells were immediately collected (400*g*, 5 min, 4 °C), washed with 1 ml ice-cold PBS and lysed in 1 ml lysis buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.2% Nonidet P40, 10% glycerol and complete protease inhibitor cocktail (Roche Biochemicals)) for 15 min on ice. The lysate was cleared twice by centrifugation at 16,000*g* in a microfuge for 10 min at 4 °C. The soluble fraction was precleared with 20 μl Sepharose-6 B (Sigma) for 2 h at 4 °C and immunoprecipitated with 20 μl protein-A–Sepharose CL-4B (Amersham) for 4 h to overnight at 4 °C. Beads were recovered by centrifugation and washed four times with 500 μl 20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.2% Nonidet P40, 10% glycerol. Beads were resupended in 600 μl 0.1 M citrate-NaOH, pH 2.7, containing 50 μg ml^{-1} lysozyme as a carrier, and incubated for 5 min at room temperature; the supernatant was precipitated with 750 μl chloroform and methanol (1:4) and centrifuged at 16,000*g* for 5 min. The upper phase was discarded and replaced with an equal volume of methanol; proteins were recovered by centrifugaton at 16,000*g* for 3 min. The pellet was redissolved by sonication in sample buffer and analysed by SDS–PAGE and western blotting. 30 μg cell lysate were analysed as an expression control. For the analysis of TRAIL-R1 and TRAIL-R2 expression, 10^8 cells were subjected to phase separation in Triton X-114 (ref. 19), precipitation with chloroform and methanol as described above, and western blotting.

Cytotoxic assay.

Cells (100 μl, 50,000 cells per well in 96-well plates) were incubated for 16 h at 37 °C, in 5% CO_2 in a humid chamber, in the presence of the indicated concentrations of sTRAIL and 1 μg ml^{-1} crosslinking mouse anti-Flag monoclonal antibody M2. Cell viability was assessed by adding 20 μl of a solution containing 2 mg ml^{-1} of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2 H-tetrazolium, inner salt (MTS) reagent (Promega) and 50 μg ml^{-1} phenazine methosulphate (PMS) to each well. Following colour development, absorbance at 490 nm was measured using an enzyme-linked immunosorbent assay (ELISA) reader.

Assay for caspase-3 activity.

Caspase-3 activity was assayed for each time point of the DISC analysis by mixing 10 μl cell lysate (30–40 μg protein) with 100 μl reaction buffer (10 mM Tris-HCl, pH 7.4, 0.1% CHAPS, 2 mM $MgCl_2$, 1 mM dithiothreitol, 5 mM EGTA, 150 mM NaCl) containing 50 μM of a fluorogenic caspase-3 substrate (Ac-DEVD-AMC; Alexis). The mixture was incubated for 60 min in an ELISA titre plate and fluorescence was measured in a Fluoroskan ELISA reader (excitation 355 nm, emission 460 nm). Caspase-3 activity is expressed as a factor of fluorescence increase relative to non-treated cells. Background was subtracted using a lysis-buffer-only control. Values are normalized with respect to protein content.

Staining with annexin-V.

Cells were stained for 15 min on ice in 50 μl annexin-V buffer containing 250 ng ml^{-1} annexin-V–fluorescein isothiocyanate (FITC) (Nexins Research, Kattendyke, The Netherlands), washed once and analysed in a fluorescence-activated cell-sorting (FACS) cytometer. Apoptosis is expressed as the percentage of annexin-V-positive cells.

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Molecular requirements for the combined effects of TRAIL and ionising radiation.

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BACKGROUND AND PURPOSE: Previously it was shown that combinatic of death ligand TRAIL and irradiation strongly increases cell kill in several human tumour cell lines. Since Bcl-2 overexpression did not strongly interfe with the efficacy, components of the mitochondrial death pathway are not required for an effective combined treatment. In the present study the minim molecular prerequisites for the efficacy of a combined treatment were determined. MATERIALS AND METHODS: Apoptosis induction in contro caspase-8 and FADD negative Jurkat cells, BJAB control and FADD-DN cel was analysed by FACS. Activation of caspase-8, -10 and -3 and cleavage of PARP was determined by immunoblotting. TRAIL receptors were activated using recombinant human TRAIL. Surface expression of TRAIL receptors DR4 and DR5 was analysed by FACS. RESULTS: Jurkat T-cells express the agonistic DR5 receptor but not DR4. Presence of FADD was found to be essential for TRAIL induced apoptosis. Caspase-8 negative cells show very low rates of apoptosis after prolonged stimulation with TRAIL. No combine effects of TRAIL with irradiation could be found in FADD-DN overexpressi and FADD deficient cells. However, the combination of TRAIL and irradiati clearly lead to a combined effect in caspase-8 negative Jurkat cells, albeit wi reduced death rates. In these cells activation of the alternative initiator caspa 10 could be detected after combined treatment. CONCLUSION: Our data sh that a combined therapy with TRAIL and irradiation will only be effective in cells expressing at least one agonistic TRAIL receptor, FADD and caspase-8 caspase-10.

MeSH Terms:

- Adaptor Proteins, Signal Transducing*
- Apoptosis/drug effects*
- Apoptosis/radiation effects*
- Carrier Proteins/metabolism
- Caspases/metabolism

- Cell Survival/drug effects
- Cell Survival/radiation effects
- Humans
- Jurkat Cells
- Membrane Glycoproteins/pharmacology*
- Receptors, Tumor Necrosis Factor/metabolism
- Tumor Necrosis Factor-alpha/pharmacology*

Substances:

- Adaptor Proteins, Signal Transducing
- Carrier Proteins
- MORT1 protein
- Membrane Glycoproteins
- Receptors, Tumor Necrosis Factor
- TNF-related apoptosis-inducing ligand
- Tumor Necrosis Factor-alpha
- death receptor-4
- death receptor-5
- Caspases
- caspase 8
- caspase 9

PMID: 12972315 [PubMed - indexed for MEDLINE]

Mitogen-Activated Protein Kinase/Extracellular Signal-Regulated Kinase Signaling in Activated T Cells Abrogates TRAIL-Induced Apoptosis Upstream of the Mitochondrial Amplification Loop and Caspase-8[1]

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Fas ligand and TNF-related apoptosis-inducing ligand (TRAIL) induce apoptosis in many different cell types. Jurkat T cells die rapidly by apoptosis after treatment with either ligand. We have previously shown that mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) can act as a negative regulator of apoptosis mediated by the Fas receptor. In this study we examined whether MAPK/ERK can also act as a negative regulator of apoptosis induced by TRAIL. Activated Jurkat T cells were efficiently protected from TRAIL-induced apoptosis. The protection was shown to be MAPK/ERK dependent and independent of protein synthesis. MAPK/ERK suppressed TRAIL-induced apoptosis upstream of the mitochondrial amplification loop because mitochondrial depolarization and release of cytochrome *c* were inhibited. Furthermore, caspase-8-mediated relocalization and activation of Bid, a proapoptotic member of the Bcl family, was also inhibited by the MAPK/ERK signaling. The protection occurred at the level of the apoptotic initiator caspase-8, as the cleavage of caspase-8 was inhibited but the assembly of the death-inducing signaling complex was unaffected. Both TRAIL and Fas ligand have been suggested to regulate the clonal size and persistence of different T cell populations. Our previous results indicate that MAPK/ERK protects recently activated T cells from Fas receptor-mediated apoptosis during the initial phase of an immune response before the activation-induced cell death takes place. The results of this study show clearly that MAPK/ERK also participates in the inhibition of TRAIL-induced apoptosis after T cell activation. ***The Journal of Immunology,*** **2002, 169: 2851–2860.**

Apoptosis or programmed cell death is important in regulating tissue homeostasis in adult organisms and during embryonic development. In the immune system, negative T cell selection, as well as termination of clonally expanded peripheral T cell populations, is conducted by apoptosis (1). Apoptosis is often initiated by external stimulation of a death receptor (DR),[4] which in turn initiates an intracellular signaling cascade, eventually leading to apoptosis. Whether the signals mediated by the activated receptors will lead to apoptosis or continued proliferation is dependent on cell type and the state of differentiation. DR responses need to be carefully regulated during different dynamic processes, such as proliferation, migration, and differentiation, to maintain an accurate size of a given cell population.

The Fas ligand (FasL) (2) and the TNF-related apoptosis-inducing ligand (TRAIL) (3, 4) are members of the TNF family. Both are able to induce rapid apoptosis in potential target cells, the sensitivity of which seems to be regulated by multiple mechanisms. Among the members of the TNF family, TRAIL shows the highest homology with the FasL. TRAIL is a 40-kDa type II transmembrane protein suggested to be involved in many biological processes, such as activation-induced death of lymphocytes (5–8), T cell-mediated cytotoxicity (9–11), and maintenance of immune-privileged sites (12), all functions that have been assigned also for the Fas receptor (FasR). While the involvement of FasR in these processes is relatively well established, further investigations are required to determine the exact role of TRAIL in these functions.

TRAIL signaling is mediated and regulated by four distinct receptors: DR4/TRAIL-R1 (13), DR5/TRAIL-R2 (14), decoy receptor (DcR)1/TRAIL-R3 (15), and DcR2/TRAIL-R4 (16), of which the DR4 and DR5 contain functional death domains and are able to induce apoptosis. In contrast, DcR1 and DcR2 act as inhibitory receptors by lacking complete death domains. The elevated expression of DcRs in normally growing tissues could possibly explain why TRAIL induces apoptosis in most transformed but not in normal cells (16, 17).

The apoptotic signaling pathway induced by ligation of the TRAIL receptors (TRAIL-R) is still fairly uncharacterized. Fas-associated death domain (FADD) (18) and caspase-8 (19) have

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[4] Abbreviations used in this paper: AICD, activation-induced cell death; CHX, cycloheximide; cyt *c*, cytochrome *c*; DAPI, 4′,6′-diamidino-2-phenylindile hydrochloride; DcR, decoy receptor; DISC, death-inducing signaling complex; DR, death receptor; ERK, extracellular signal-regulated kinase; FADD, Fas-associated death domain; FasL, Fas ligand; FasR, Fas receptor; GS, goat serum; HA, hemagglutinin; MAPK, mitogen-activated protein kinase; MKK1, MAPK kinase 1; MMP, mitochondrial membrane potential; PKC, protein kinase C; tBid, truncated Bid; TPA, tetradecanoyl phorbol acetate; TMRM, tetramethyl rhodamine methyl ester; TRAIL, TNF-related apoptosis-inducing ligand; TRAIL-R, TRAIL receptor.

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been previously established as important components in the FasR death-inducing signaling complex (DISC) (20). In this sense, both FADD and caspase-8 have also been indicated as crucial elements in the TRAIL-mediated signaling machinery (21–23). Activation of caspase-8 in the DISC results in activation of downstream caspases and cleavage of cytosolic substrates such as Bid (24). Bid engages a mitochondrial amplification pathway, which has been suggested to be required for induction of apoptosis in some cell types. The cleaved or truncated Bid (tBid) (25) translocates to the mitochondria, where it triggers depolarization of the mitochondria. In concert with the altered mitochondrial membrane potential (MMP), cytochrome *c* (cyt *c*) is released to the cytosol, where it forms the apoptosome together with apoptosis protease-activating factor 1 and caspase-9 (26). In turn, caspase-9 can activate downstream caspase-3 or boost the activation of other caspases, such as caspase-8, to complete the mitochondrial amplification loop (reviewed in Ref. 27). The relative importance of the mitochondrial amplification loop in DR-mediated apoptosis is still not fully understood, and both mitochondria-dependent and -independent activation mechanisms have been identified. The available information on the involvement of the mitochondrial activation in TRAIL-R signaling is very scarce.

Suppression of apoptosis has been shown to be of major importance during many physiological as well as pathological processes. Apoptosis can be negatively regulated by inhibitor proteins, such as Bcl family proteins (reviewed in Ref. 28), FLIPs (reviewed in Ref. 29), or inhibitors of apoptosis protein (reviewed in Ref. 30). Another mode of regulation is through expression of DcRs, whose presence has been described for both the Fas (31) and the TRAIL-R system (reviewed in Ref. 32). Finally, protein kinase-mediated signaling has been described as an effective way of directing DR signals (reviewed in Ref. 33). In contrast to regulation by inhibitor proteins and DcRs, phosphorylation-based signaling occurs without requirement of newly synthesized proteins. In this respect, especially the classical mitogen-activated protein kinase (MAPK) signaling pathway has been implicated as a dominant negative regulator of DR-mediated apoptosis. We have observed that MAPK/extracellular signal-regulated kinase (ERK) signaling potently modifies FasR responses (34, 35) and found indications that it is involved in regulating also TRAIL-R responses (36). We have also shown that MAPK/ERK signaling from the TCR is able to protect T cells from FasR-mediated apoptosis (37) before they commit activation-induced cell death (AICD). Therefore, we wanted to test whether this type of regulation could also apply for the TRAIL-Rs. The results of the present study show that the MAPK/ERK pathway in activated Jurkat T cells suppresses TRAIL-mediated apoptosis in a similar fashion as it suppresses FasR-mediated apoptosis. Because little was known about the role of the mitochondrial amplification loop in TRAIL-R-mediated signaling, we paid special attention to clarifying where the inhibition takes place in relation to the proapoptotic mitochondrial signaling sequence. Our results show that MAPK/ERK abrogates the apoptotic signal upstream of the mitochondrial amplification loop by inhibiting initiator caspase activity. This mechanism could especially be involved in regulation of the persistence of peripheral T cell populations.

Materials and Methods

Cell culture

The human leukemic T cell line Jurkat (clone E6-1) was received from American Type Culture Collection (Manassas, VA). The cells were cultured in RPMI 1640 medium supplemented with 10% FCS, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C in 5% CO_2 in air. The cells were kept at a density of 0.5–1.0 × 10^6/ml.

Jurkat T cells were incubated at a density of 1 × 10^6/ml with TRAIL (100 ng/ml; Alexis, Läuflingen, Switzerland) along with 2 μg/ml cross-linking FLAG-tagged Ab M2 (Sigma-Aldrich, St. Louis, MO) or 100 ng/ml agonistic anti-human FasR IgM Ab (MBL, Watertown, MA) for the indicated time periods in the absence or presence of 100 μg/ml immobilized OKT3 (R.W. Johnson Pharmaceutical Institute, Bassersdorf, Switzerland) as described earlier (37), 20 nM tetradecanoyl phorbol acetate (TPA; Sigma-Aldrich), 30 μM PD 98059 (Calbiochem, La Jolla, CA), or 5 μM cycloheximide (CHX; Sigma-Aldrich).

Analysis of phosphatidylserine exposure

To detect phosphatidylserine exposure by flow cytometry, Jurkat T cells were washed once with PBS and incubated for 10 min in 400 μl binding buffer (2.5 mM HEPES/NaOH (pH 7.4), 35 mM NaCl, 0.625 mM $CaCl_2$) with 1 μl annexin V-FITC (Alexis) and analyzed on a FACScan flow cytometer (BD Biosciences, San Jose, CA) or viewed under a RMB epifluorescence microscope (Leica, Deerfield, IL).

Immunoblotting techniques used

Immunoblotting was performed by lysing cells in Laemmli sample buffer and then resolving the proteins on a 12.5% SDS-PAGE. The separated proteins were transferred to a nitrocellulose membrane (Schleicher & Schuell, Dassel, Germany) probed with the specific Ab to ERK2 (BD Transduction Laboratories, Lexington, KY), phospho-ERK1/2 (New England Biolabs, Boston, MA), caspase-8 (a kind gift from P. Krammer, German Cancer Research Center, Heidelberg, Germany) (38), Bid (Santa Cruz Biotechnology, Santa Cruz, CA), Hsc70 (StressGen Biotechnologies, Victoria, British Columbia, Canada), or actin (Sigma-Aldrich), followed by coupling to the appropriate HRP-conjugated secondary Abs and visualization with the ECL system (Amersham, Little Chalfont, U.K.)

Transfection studies

Cells were transiently transfected by electroporation (220 V, 975 μF) in 400 μl of OptiMeM (Life Technologies, Rockville, MD) and allowed to rest for 48 h before treatments. The DNA constructs used were pMCL-HA-MKK1-K97 M and pMCL-HA-MKK1-S218E/S222, encoding for hemagglutinin (HA)-tagged dominant negative and constitutively active forms of MAPK kinase 1 (MKK1). The plasmid was a kind gift from N. Ahn (University of Colorado, Boulder, CO). Mock transfections were conducted using a pIRES-EGFP plasmid (Clontech Laboratories, Palo Alto, CA). For detection of transfected cells, the cells were fixed with 3% formaldehyde in PBS and permeabilized with 0.1% Triton X-100 (Sigma-Aldrich). After washing and blocking, cells were incubated with 10 μg/ml of a monoclonal HA-specific Ab (12CA5; Boehringer Mannheim, Mannheim, Germany) followed by incubation with FITC-conjugated anti-mouse secondary Ab and 10 mg/ml Hoechst 33342 (Molecular Probes, Eugene, OR). Bid-GFP transfection studies were conducted as mentioned above. The plasmid was a kind gift from G. J. Gores (Mayo Clinic, Rochester, MN) (39). For detection of apoptotic nuclei, cells were labeled with Hoechst 33342. Cells were finally mounted in 50% glycerol and viewed under a Leica RMB epifluorescence microscope.

Measurement of MMP by confocal microscope

To measure MMP, Jurkat T cells were equilibrated with 50 nM tetramethyl rhodamine methyl ester (TMRM; Molecular Probes) in RPMI 1640 medium supplemented with 25 mM HEPES (pH 7.2) for 1 h at 37°C in the dark. Subsequently, TRAIL was added to the equilibration medium. Leica TCS SP confocal microscope with 63× NA 1.4 oil immersion planapochromat objective was used to collect TMRM and transmission images at given time points. Red fluorescence of TMRM was imaged by using 568 nm excitation light from argon/krypton laser and emitted light was collected through 575–705 nm.

cyt c immunofluorescence analysis by confocal microscope

For immunofluorescence analysis, Jurkat cells were centrifuged onto glass coverslips, washed with PBS, and fixed with 3% paraformaldehyde. Subsequently, cells were permeabilized with 0.5% Triton X-100/PBS for 10 min at room temperature. After blocking with normal goat serum (GS), samples were incubated with mouse anti-cyt *c*, (clone 6H2.B4, 1:150 in PBS/0.01% Triton X-100 with 1.5% GS; BD PharMingen, San Diego, CA) for 2 h in a humidified dark chamber at 37°C. After three washes with PBS/0.01% Triton X-100, samples were incubated with Alexa 488-conjugated goat anti-mouse IgG (1:150 in PBS/0.01% Triton X-100 with 1.5% GS; Molecular Probes) for 45 min in a dark chamber. After three washes with PBS/0.01% Triton X-100, nuclei of the cells were counterstained with

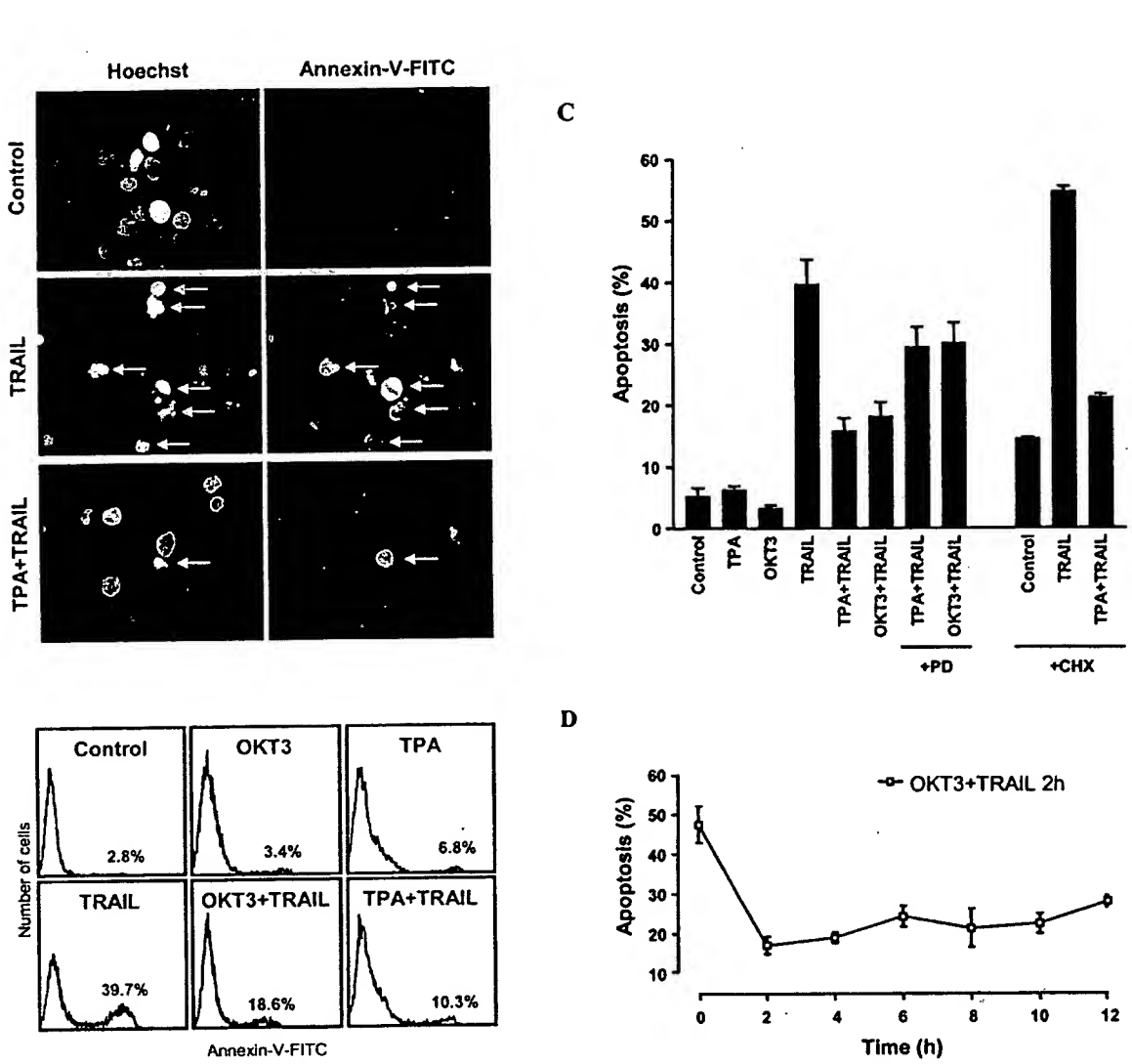

FIGURE 1. TRAIL-induced apoptosis is inhibited by TPA and OKT3 through a MAPK/ERK-dependent mechanism that is independent of protein synthesis. *A*, Jurkat T cells were stimulated with or without TPA (20 nM), TRAIL (100 ng/ml and 2 μg/ml oligomerizing Ab M2), or TPA and TRAIL. After 2 h the cells were labeled with annexin V-FITC and Hoechst 33342 for detection of apoptotic cells with fluorescence microscopy. Arrows indicate the apoptotic cells. *B*, The histograms show quantification of the number of apoptotic cells after the indicated treatments. Cells were labeled with annexin V-FITC and analyzed using a FACScan flow cytometer. Percentages of apoptotic cells with phosphatidylserine exposure are shown. *C*, Jurkat T cells were preincubated with 20 nM TPA (10 min) or immobilized OKT3 (30 min) before addition of 100 ng/ml TRAIL and 2 μg/ml of an oligomerizing Ab (M2). After 2 h the proportion of apoptotic cells was determined by FACS analysis of annexin V-FITC-labeled cells. Bars indicate the percentage of cells with exposed phosphatidylserine. Pretreatment of cells with 30 μM PD 98059 (30 min) before incubation with TPA, OKT3, and TRAIL abrogated the protective effect of TPA and OKT3. To study whether the observed protection from TRAIL-induced apoptosis is protein synthesis independent, we pretreated Jurkat T cells for 15 min with 5 μM CHX before the addition of TPA and/or TRAIL. *D*, OKT3 suppressed TRAIL-induced apoptosis for 12 h. Cells were incubated with immobilized OKT3 for the indicated time points and then stimulated with TRAIL for 2 h and analyzed as in *C*. The data represent mean values (mean ± SEM) from a minimum of three separate experiments.

0.1 μg/ml 4′,6′-diamidino-2-phenylindile hydrochloride (DAPI) and coverslips were mounted on microscope slides in 80% glycerol in PBS. cyt *c* release and nuclear morphology of the cells were imaged by Leica TCS SP MP confocal microscope with 63× NA 1.4 oil immersion planapochromat objective. Alexa 488 fluorescence was excited by using a 488-nm excitation line from argon/krypton laser and emission window was set at 492–560 nm. DAPI fluorescence was imaged by using a 780-nm excitation light from Ti-Sapphire (Tsunami; Spectra Physics, Mountain View, CA) laser and emission light was recorded through 400–490 nm.

Surface expression analysis of DR4 and DR5

A total of 0.5 × 10^6 cells were treated with TPA for the indicated time points with or without TRAIL. After washing, cells were blocked for 30 min with 1% BSA in PBS. Cells were then incubated with 1 μg of Abs to DR4 or DR5 (Alexis) in 1% BSA in PBS for 30 min followed by washing with PBS. Finally, cells were incubated with Alexa 488-conjugated goat anti-mouse IgG (Molecular Probes) for 30 min. After washes cells were analyzed on a FACScan flow cytometer. Only secondary Ab was used as a control.

TRAIL-R immunoprecipitation and DISC analysis

A total of 2 × 10^8 Jurkat cells per sample were left untreated or pretreated with 20 nM TPA in a 37°C water bath at cell densities between 1 and 2 × 10^6/ml. After 15 min cells were pelleted at 500 × *g* for 7 min and resuspended in 1 ml prewarmed RPMI medium. To stimulate TRAIL-Rs, 1 μg FLAG-tagged recombinant human soluble TRAIL (Alexis) and 2 μg anti-FLAG monoclonal M2 Ab (Sigma-Aldrich) were added to the cell suspension. Cells were incubated in a 37°C water bath for 15 min and the reaction was stopped by adding 10 ml of ice-cold PBS to the cell suspension. Cells were pelleted, washed with ice-cold PBS, and lysed in 1 ml lysis buffer (20

mM Tris-HCl (pH 7.4), 150 mM NaCl, 10% glycerol, 0.2% Nonidet P-40, 0.1% deoxycholate, and complete protease inhibitor mixture (Roche, Basel, Switzerland) for 30 min on ice. The cell debris was removed by centrifugation at 15,000 × *g* for 15 min at 4°C. The amount of protein was determined by Bradford assay and an equal amount of protein from each sample was precleared with 50 μl of Sepharose-CL-4B for 2 h at 4°C. A total of 5 μg of monoclonal anti-DR5 and 2.5 μg monoclonal anti-DR4 (Alexis) were added to samples and immunoprecipitated with 15 μl protein G beads (Amersham) for 2.5 h at 4°C. Beads were washed six times in 1 ml lysis buffer, resuspended in 3× Laemmli sample buffer, and boiled for 3 min. About one-third of immunoprecipitation samples and 20–50 μg protein from cell lysates were analyzed by 12.5 or 10% SDS-PAGE. Western blot was performed with anti-DR5 (Alexis), anti-FADD (BD Transduction Laboratories), caspase-8 (C15 caspase-8 Ab, a kind gift from P. Krammer, German Cancer Research Center), and anti-FLIP (Alexis) as described above.

Results

TPA and OKT3 suppress TRAIL-induced apoptosis through a MAPK/ERK-dependent mechanism

To study whether MAPK/ERK activation is able to modulate TRAIL-induced apoptosis of Jurkat T cells, we pretreated cells with two known MAPK/ERK activators (34, 37), the phorbol ester TPA or OKT3, the latter of which is an agonistic Ab to CD3 of the TCR complex. Our results show that pretreatment with both TPA and OKT3 suppresses TRAIL-induced apoptosis (Fig. 1, *A–C*). Apoptosis was measured by flow cytometric analysis of phosphatidylserine exposure on the cell membrane with annexin V conjugated to FITC (Fig. 1, *B* and *C*). Incubation with TRAIL alone induced rapid apoptosis in the cells. After 2 h almost 50% of the cells were apoptotic, whereas pretreatment with TPA or OKT3 efficiently suppressed TRAIL-induced apoptosis, as indicated by decreased phosphatidylserine exposure (Fig. 1, *A* and *B*) and DNA fragmentation (data not shown). Furthermore, the T cell activator OKT3 was able to suppress TRAIL-induced apoptosis for at least 12 h (Fig. 1*D*). These results on inhibition of TRAIL-induced apoptosis by MAPK/ERK activators correspond well to our previous results showing that MAPK/ERK signaling is an effective inhibitor of FasR-mediated apoptosis (34), as well as our data showing that activated T cells stay insensitive to FasR-mediated apoptosis as long as their MAPK/ERK activity is elevated (37).

To corroborate the assumption that MAPK/ERK activity could be involved in the observed suppression of TRAIL-induced apoptosis, we tested whether pretreatment with the specific MKK1 inhibitor, PD 98059, could abolish the protective effect of OKT3 and TPA. Our results clearly show that pretreatment with PD 98059 abolished the protective effect of both TPA and OKT3 (Fig. 1*C*), thus indicating that the protective effect of these compounds is MAPK/ERK dependent. Also, in agreement with previous observations on the FasR (34), the protective effect was protein synthesis independent as the MAPK/ERK-mediated protection was equally efficient in the presence of CHX (Fig. 1*C*). To verify that OKT3 and TPA stimulation induces phosphorylation of MAPK/ERK, we analyzed the activation of MAPK/ERK by immunoblotting with an Ab that recognizes phosphorylated MAPK/ERK (Fig. 2). Incubation with TPA and OKT3 induced an increase in MAPK/ERK activity, which was inhibited by pretreatment with PD 98059 (Fig. 2), correlating well with the relative inhibition of apoptosis observed in Fig. 1*C*. The results further support that the protective effect of TPA and OKT3 is MAPK/ERK dependent.

Constitutively active MKK1 protects Jurkat T cells from TRAIL-induced apoptosis

The suppressive role of MAPK/ERK in TRAIL-induced apoptosis was verified by transient transfections with HA-tagged constitutively active and dominant negative mutants of MKK1 before

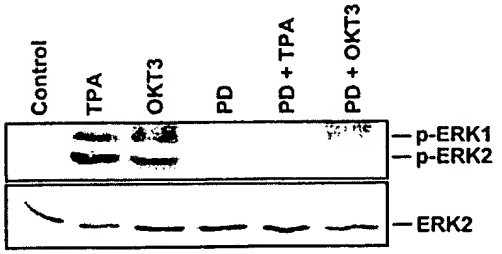

FIGURE 2. TPA and OKT3 induce MAPK/ERK activation in Jurkat T cells. Treatment of cells with TPA (10 min) and OKT3 (30 min) induces increased MAPK/ERK phosphorylation, which is inhibited by pretreatment with PD 98059 as shown by immunoblotting with a phosphospecific MAPK Ab. The *lower panel* shows equal loading of ERK in all samples. A representative immunoblot from three experiments is shown.

treatment with TRAIL. The transfected cells were then visualized by immunofluorescence labeling of HA and by DNA labeling with Hoechst 33342 to identify the nuclear morphology of the cells (Fig. 3). Constitutively active MKK1 (MKK1-CA) rendered the cells insensitive to apoptosis induced by TRAIL, while this effect was lost when cells were transfected with the dominant negative mutant. Mock transfections with green fluorescent protein did not affect the number of apoptotic cells.

Activation of MAPK/ERK does not affect the levels of DR4 and DR5 on the cell surface

Sensitization to DR-mediated apoptosis could be modulated by altered surface expression of the receptors. To rule out the possibility of surface receptor down-regulation, we analyzed the relative amount of DR4 and DR5 on the surface of Jurkat T cells. Jurkat T cells were immunofluorescence labeled with mAbs to the two respective receptors and analyzed by flow cytometry. The results show that predominantly DR5 is expressed on Jurkat T cells and that MAPK/ERK activation does not affect the relative number of receptors on the cell surface after treatment with TPA for up to 2 h (Fig. 4). The amount of DR4 and DR5 on the cell surface did not change in the presence of TRAIL.

MAPK/ERK signaling inhibits release of cyt c, loss of MMP, and translocation of tBid

To study at what level in the apoptotic activation machinery the inhibitory effect of MAPK/ERK is targeted, we started by analyzing whether the mitochondrial amplification loop is affected. To assess this question, we analyzed changes in the MMP after treatment with TRAIL alone or after pretreatment with TPA. While the cells that were treated with TRAIL alone lost their MMP, cells pretreated with TPA were not affected at this level (Fig. 5*A*). Also, in FasR-mediated apoptosis the MMP was lost (data not shown). The FasR-mediated decrease in MMP was also abolished by the TPA-mediated activation of MAPK/ERK (data not shown). To show that this was a MAPK/ERK-dependent effect, we pretreated the cells with the MKK1 inhibitor PD 98059. Inhibition of MAPK/ERK reinduced the loss in MMP and accelerated apoptosis. Because decreased MMP has been suggested to cause release of cyt *c* to the cytosol (40), it was to be expected that cyt *c* release would also be affected by activation of MAPK/ERK. Jurkat T cells treated with TRAIL alone or pretreated with TPA were immunolabeled for cyt *c* and viewed under a microscope. In control cells cyt *c* was located in the mitochondria, which can be seen as clusters next to the nucleus in Fig. 5*B*. In apoptotic Jurkat T cells, treated with TRAIL alone for 2 h, TRAIL-caused release of cyt *c* from the mitochondria to the cytosol was clearly visible. When

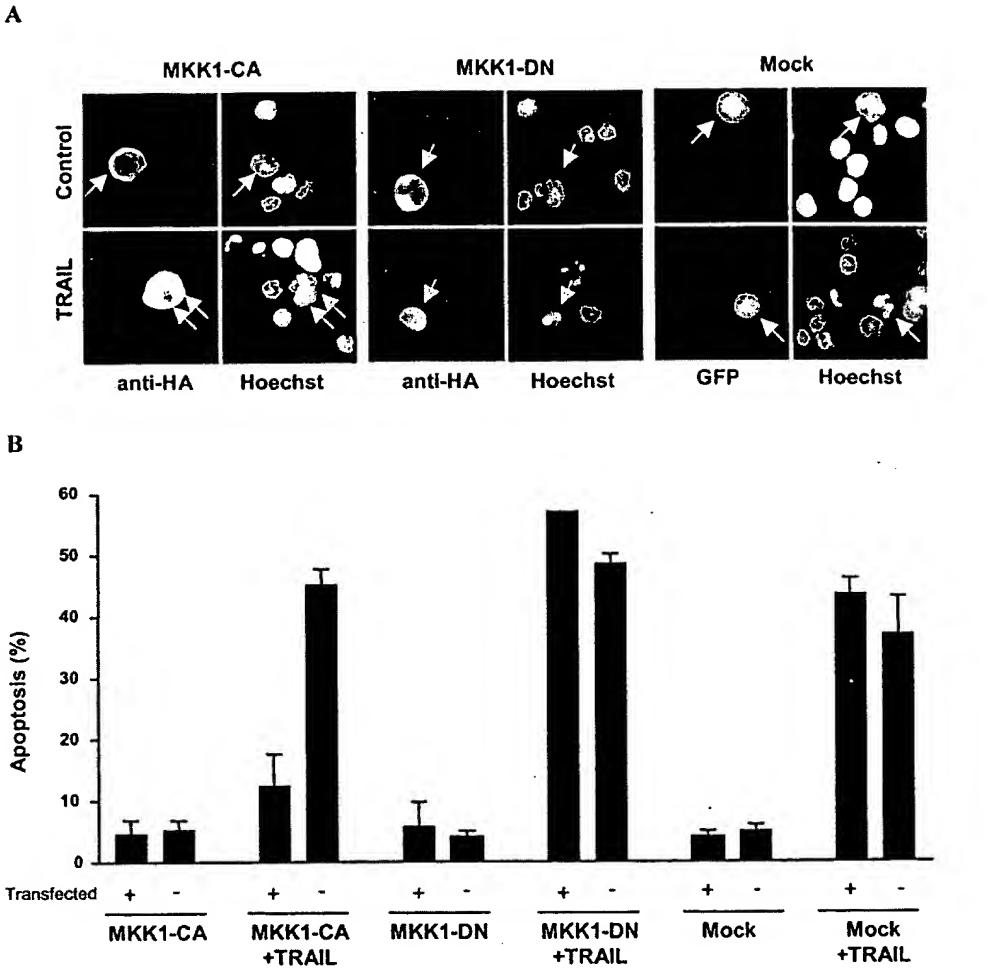

FIGURE 3. MKK1-CA protects Jurkat T cells from TRAIL-induced apoptosis. *A*, Representative immunofluorescence micrographs of cells transiently transfected with MKK1-CA and MKK1-DN and then treated with or without TRAIL for 2 h are shown. Nuclear alterations were visualized by Hoechst 33342 labeling and transfected cells by immunofluorescence labeling of HA. Mock-transfected cells were treated as indicated above with or without TRAIL stimulation. Arrows indicate the transfected cells. *B*, The percentage of apoptotic cells was counted among transfected and untransfected cells. The data represent mean values (mean ± SEM) from a minimum of three separate transfections.

cells were pretreated with the MAPK/ERK activator TPA, the release of cyt *c* was inhibited. Also in this case, treatment with PD 98059 reversed this inhibition and allowed the cells to undergo apoptosis. Also, FasR-mediated cyt *c* release was inhibited (data not shown). Treatment with TPA or PD alone did not affect the release of cyt *c*.

Decrease in MMP and release of cyt *c* from the mitochondria have been shown to be initiated by the translocation of a 15-kDa tBid from the cytosol to the mitochondria (reviewed in Ref. 41). To address the question of whether MAPK/ERK activation affects translocation of tBid, we transiently transfected Jurkat T cells with Bid-GFP. Bid-GFP Jurkat T cells were then treated with TRAIL. In control cells, Bid-GFP showed a diffuse cytoplasmic distribution (Fig. 5*C*). After 2 h in the presence of TRAIL all Bid-GFP in apoptotic cells was located in clusters near the nucleus that could be referred to as mitochondria. When the transfected cells were pretreated with TPA, Bid-GFP was no longer detected in clusters and apoptosis was inhibited. Instead, Bid was diffusely located in the cytosol similarly to control cells. Pretreatment with PD 98059 abolished the inhibition of Bid-GFP translocation (Fig. 5*C*) as well as subsequent apoptosis (Fig. 1*C*). Again, PD or TPA had no effect alone.

Taken together these results show that TRAIL-induced apoptosis is directed toward the mitochondrial amplification loop and that MAPK/ERK signaling protects Jurkat T cells from apoptosis by inhibiting any dysregulation of the mitochondria by turning off the mitochondrial amplification loop.

MAPK/ERK activation suppresses the cleavage of Bid and caspase-8

To examine whether the protective effect of MAPK/ERK signaling would occur at the level of the DISC, we analyzed how the cleavage of initiator caspase-8 was affected in cells treated with TPA before incubation with TRAIL. The results in Fig. 6*A* show that the cleavage of caspase-8 to the active 18-kDa fragment is markedly reduced in cells pretreated with TPA. Also, processing to the intermediate 42/43-kDa fragments is reduced in the presence of TPA. Caspase-8 has previously been shown to activate Bid by cleavage to a 15-kDa tBid fragment (25). Therefore, we wanted to examine whether activation of Jurkat T cells suppresses the cleavage of Bid to its active proapoptotic 15-kDa fragment. As expected, its cleavage was reduced after preincubation of the cells with TPA (Fig. 6*B*). This inhibition is likely to prevent the advancing of the apoptotic signal to the mitochondrial amplification loop, which has been implicated to be necessary for FasR-mediated apoptosis in type II cells (42).

Taken together, the results show that MAPK/ERK activation inhibits TRAIL-induced apoptosis at the early stages of the apoptotic machinery before involvement of the mitochondria.

Activation of MAPK/ERK does not affect the recruitment of FADD and caspase-8 to the TRAIL-DISC

To examine whether the observed protection mediated by MAPK/ERK activation could be located at the very early stages of DR

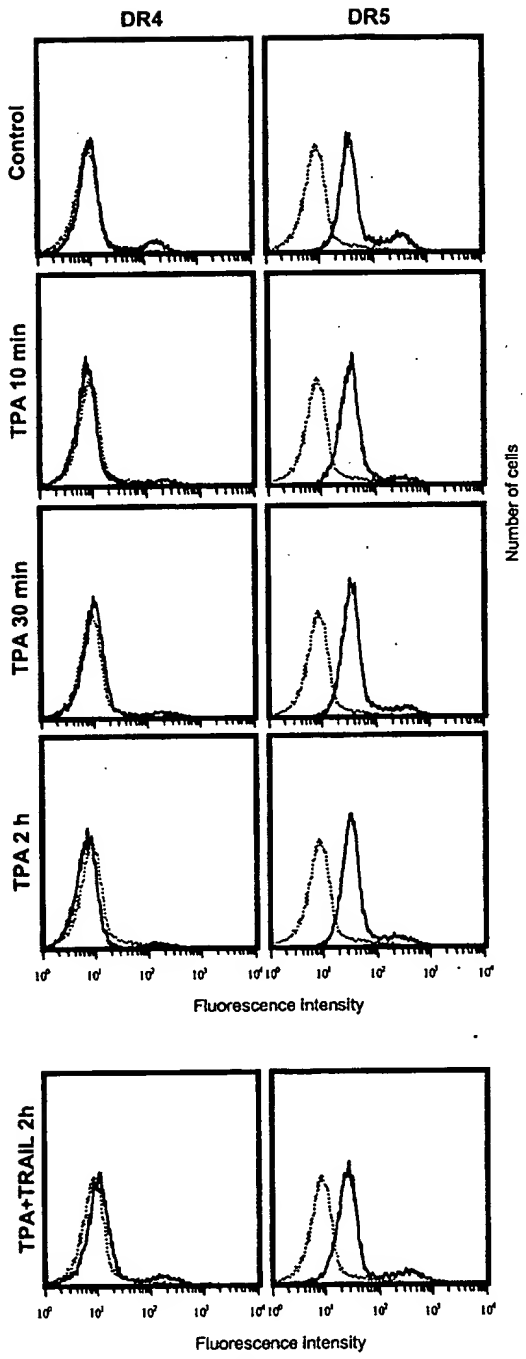

FIGURE 4. The protective effect of MAPK/ERK activation is not caused by cell surface death receptor down-regulation. The surface expression of the TRAIL-Rs DR4 and DR5 were not altered during activation of Jurkat T cells. Cells were treated with TPA alone for the indicated time points or in the presence of TRAIL for 2 h. Cells were labeled with mAbs to DR4 or DR5 and analyzed on a FACScan flow cytometer. Secondary Ab was used as a control (dashed line).

signaling, we immunoprecipitated the TRAIL-DISC to analyze the assembly of the adapter proteins recruited to the DISC. Because Jurkat T cells have been indicated to be type II cells, only a moderate amount of DISC is formed after receptor activation, also reflected in our experiments by the relative low levels of both FADD and caspase-8 coimmunoprecipitated with DR4 and DR5 after TRAIL stimulation (Fig. 7*A*). To control successful immunoprecipitation, the presence of DR5 in the immunoprecipitates was detected in the immunoprecipitated samples but not in the protein G control devoid of immunoprecipitating Abs. In our study we did not detect any changes in the amount of FADD or caspase-8 recruited to the DISC after pretreatment with TPA (Fig. 7*A*). Although the overall caspase-8 cleavage is reduced by MAPK/ERK activation (Fig. 6*A*), the amount of caspase-8 cleaved at the DISC is equal in the presence of TPA. Furthermore, we did not detect any changes in the recruitment of cFLIP to the DISC upon TPA stimulation (data not shown). To verify that MAPK/ERK was active during the same experiment, we also analyzed the cell lysates for phosphorylated ERK1/2. Active ERK1/2 could be detected only in the presence of TPA (Fig. 7*B*). Furthermore, to verify the protecting effect of TPA under the same conditions, cells were further incubated at 37°C and later monitored for apoptosis. The cells pretreated with TPA were still protected from TRAIL-induced apoptosis after several hours (data not shown). Together these experiments show that the assembly of the TRAIL-DISC is not affected by the elevated MAPK/ERK activity as earlier shown also for the FasR-DISC (37).

Discussion

Although the functions and physiological roles of TRAIL-Rs are not by any means as well established as those of the FasR, it is quite obvious that the emerging view of TRAIL-R tasks converges with the established roles of the FasR/FasL system, as listed in the introduction. While many features of the TRAIL-Rs are shared by the FasR and because TRAIL shows high similarity with the FasL, it is not surprising that the physiological roles of TRAIL-Rs resemble those of FasR. In addition to the converging physiological roles, there are several recent studies to indicate also that the regulation of both receptor systems is executed by similar mechanisms. Both receptor systems mediate their signals through the assembly of a DISC (20) that holds FADD (18) and caspase-8 (19) as key apoptotic signal transducers. The signaling of both TRAIL-R and FasR are regulated by DcRs and by regulation of receptor levels present on the cell surface (31, 32), and in the cytoplasmic domains FLIP has been implicated as both a signal regulator (43, 44) and a signal conveyor (45). In addition to these shared regulatory mechanisms, all of which work at the level of protein-protein interactions, we obtained in the present study compelling evidence that MAPK/ERK is a dominant inhibiting regulatory mechanism that directs TRAIL-R signaling in the same way as FasR signaling. Similarly to the FasR, MAPK/ERK regulates TRAIL-R responses through direct posttranslational regulation, without involvement of newly synthesized proteins. This type of direct signaling-mediated regulation is likely to act in concert with the other described regulatory mechanisms and would be of special importance in dynamic situations when rapid protection or sensitization is required but there is no time to engage the transcriptional machinery or to regulate protein levels otherwise.

In T cells both FasR and FasL are up-regulated upon T cell activation and both participate later in the attenuation of immune responses, to avoid formation of autoreactive T cells (reviewed in Ref. 46). This modulation occurs through AICD, where T cells kill themselves or their neighboring cells, supposedly by activating the FasR. However, T cells are insensitive to FasR-mediated apoptosis immediately after activation, although they express both FasR and FasL. This insensitivity is important in order for the activated T cells to fulfill their task of killing the target cell. It has also been shown that TRAIL is up-regulated immediately after T cell activation (47, 48) and that TRAIL later participates in the down-regulation of immune responses. While it is still quite controversial whether TRAIL is involved in AICD of human peripheral T lymphocytes in vivo (5, 7, 8), there are results indicating that AICD of Jurkat T cells (8) as well as T cells derived from HIV patients (6)

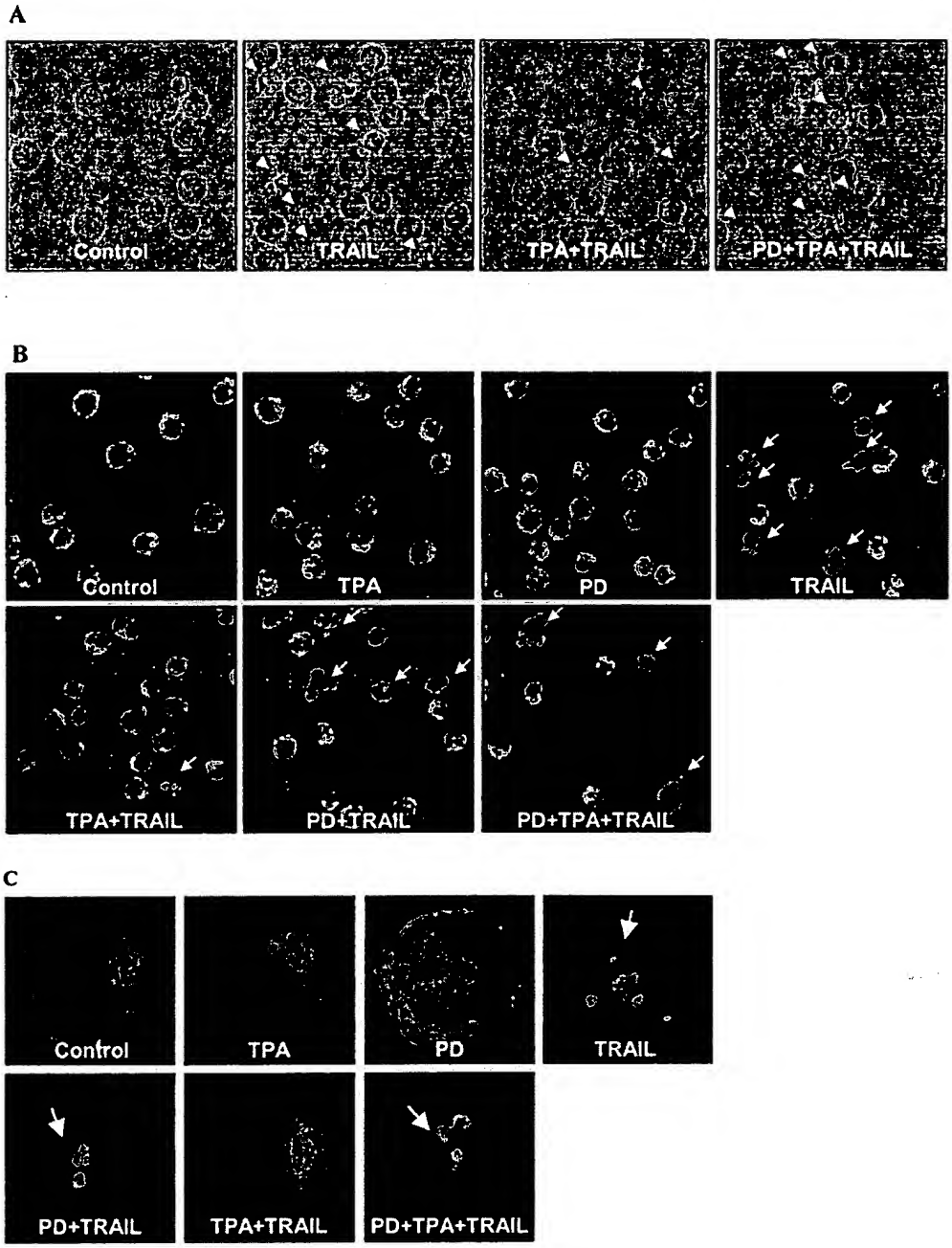

FIGURE 5. *A*, MAPK/ERK activation inhibits depolarization of mitochondria. Shown are confocal laser scanning micrographs of Jurkat T cells labeled with MMP dye TMRM and then treated as indicated. After 2 h live cells were analyzed by confocal microscopy. Apoptotic cells were identified by morphology from transmission images (arrowheads). Note that the apoptotic cells have lost their MMP as determined by decreased TMRM fluorescence intensity. *B*, Activation of MAPK/ERK inhibits the release of cyt *c*. Shown are confocal laser scanning micrographs of Jurkat T cells incubated for 2 h as indicated. Cells were labeled for cyt *c* (signal shown in green) and DNA was labeled with DAPI (signal shown in red). Note that the apoptotic cells (arrows) show cytoplasmic localization of cyt *c* compared with the clustered mitochondrial localization in the control and TPA-treated cells. *C*, MAPK/ERK activation prevents tBid translocation to mitochondria. Jurkat T cells were transfected transiently with GFP-tagged Bid. Cells were then treated as indicated and analyzed by fluorescence microscopy to detect the localization of Bid-GFP. Activation of MAPK/ERK by TPA prevented Bid-GFP translocation to the mitochondria and apoptosis. Arrows point out the apoptotic Bid-GFP transfected cells.

involves TRAIL. There are also indications that TRAIL would inhibit cell cycle progression, thereby arresting the T cells so they can be killed by other ligands, such as FasL (49). Therefore, it is important that recently activated T cells stay insensitive to both FasL and TRAIL during the early phase of activation so that the cells can fulfill their task of killing their target cells without being sensitive to their own death ligands.

MAPK/ERK signaling modulates apoptosis induced by TRAIL

Because our previous results indicate that the insensitivity of activated T cells to FasR-mediated apoptosis depends on MAPK/ERK activation (34, 37), we examined whether MAPK/ERK signaling could suppress TRAIL-induced apoptosis during early phases of T cell activation. Our results show that activated Jurkat T cells display similar kinetics of MAPK/ERK activation and

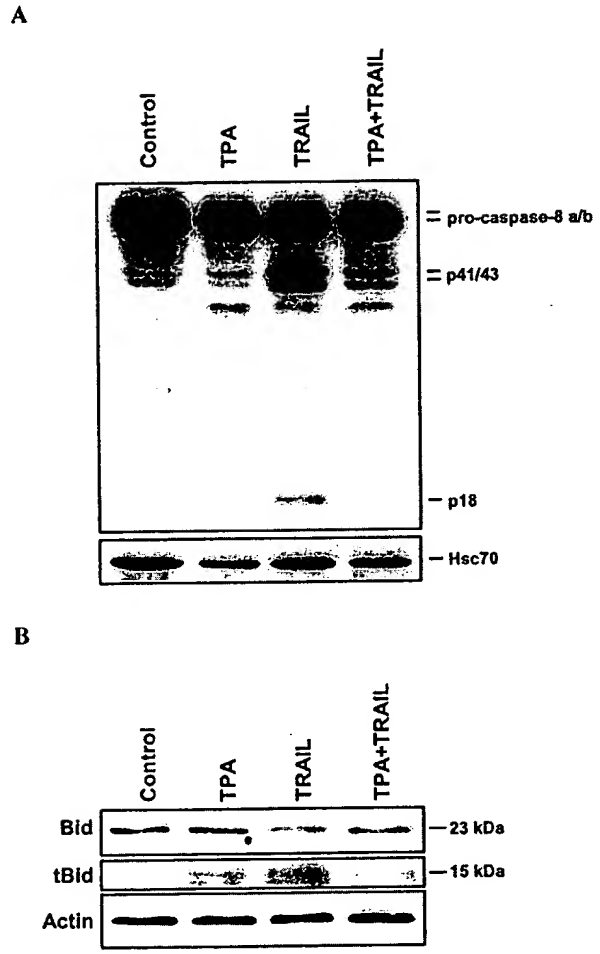

FIGURE 6. MAPK/ERK activation suppresses cleavage of caspase-8 and Bid. Jurkat T cells were treated as indicated before assessment of caspase-8 (*A*) and Bid (*B*) cleavage by Western blotting with specific Abs. Caspase-8 activation can be observed as the appearance of an 18-kDa active fragment, whereas activation of Bid is detected by the appearance of tBid as a 15-kDa fragment. TPA treatment inhibited the cleavage of both caspase-8 and Bid, thereby preventing their activation. The *lower panels* show equal loading of Hsc70 and actin in all samples. Representative immunoblots from three experiments are shown.

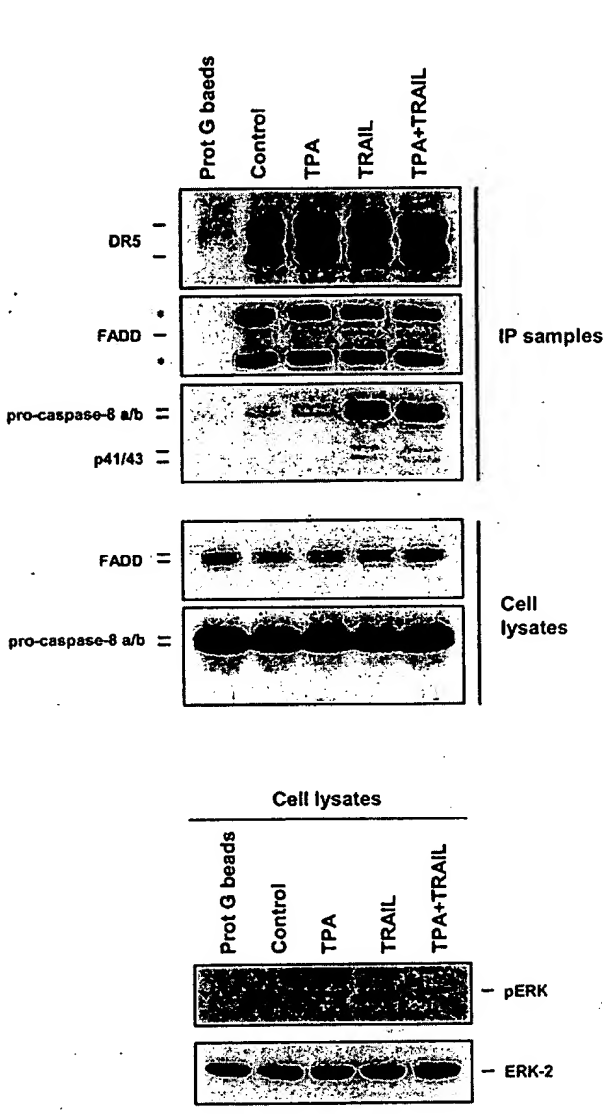

FIGURE 7. MAPK/ERK activation does not affect the TRAIL-DISC. Jurkat T cells were pretreated with or without 20 nM TPA and then stimulated with TRAIL for 15 min. Then the TRAIL-DISC was immunoprecipitated with mAbs to DR4 and DR5. To rule out possible unspecific binding of proteins during immunoprecipitation, an equal amount of cell lysate was incubated with protein G beads without any immunoprecipitating Abs (prot G beads). *A*, The immunoprecipitates were resolved on a SDS-PAGE and analyzed for the presence of DR5, FADD, and caspase-8. Cell lysates were also analyzed for FADD and caspase-8 to show equal input of protein. Nonspecific IgG reactivity is marked with an asterisk. *B*, The cell lysates were also analyzed for the presence of phosphorylated MAPK/ERK (pERK) to show that MAPK/ERK was activated during the experiment. Representative immunoblots from two separate experiments are shown.

insensitivity to TRAIL, as was shown with the FasR (37). Our results indicate that MAPK/ERK signaling mediates a protective signal to both FasR-mediated and TRAIL-induced apoptosis during these early phases of T cell activation. This protective signal is then turned off at the end of the immune response to allow AICD and attenuation of the immune response. There is one report indicating that activation of protein kinase C (PKC) can protect cells from TRAIL-induced apoptosis independently of MAPK/ERK (50). This study indicated that PKC activity is mainly responsible for the observed protection from TRAIL-induced apoptosis upon TPA treatment. Differences in the experimental setup could explain the different outcome of the experiments. It is also difficult to separate these two pathways at the level of PKC, because it is an upstream regulator of MAPK/ERK, especially by using only pharmacological signaling inhibitors and activators, the principal approach in the above-mentioned study. There is also the distinct possibility that PKC and MAPK/ERK act as separate signaling entities regulating DR responses. However, while it is plausible that PKC and possibly other signaling modulators regulate FasR and TRAIL-R sensitivity, our results undoubtedly show that the MAPK/ERK signaling pathway can function as a single dominant regulator of TRAIL responses.

The MAPK/ERK-mediated protection is independent of protein synthesis and does not alter the relative amount of DR4 or DR5 on the cell surface

It is well known that several cell lines can be sensitized to DR-mediated apoptosis by pretreatment with protein synthesis inhibitors (36). This raises the possibility that the MAPK/ERK-mediated effect could be protein synthesis dependent. However, our previous results have demonstrated that MAPK/ERK-mediated suppression

of FasR-mediated apoptosis is not protein synthesis dependent (34–36). The results presented in this work show that the same is true for MAPK/ERK-mediated suppression of TRAIL-induced apoptosis. Thus, a high MAPK/ERK activity is sufficient to trigger the protective effect. Direct modulation by phosphorylation-based signaling is beneficial to quickly modulate TRAIL sensitivity in situations with rapidly fluctuating conditions, such as cell growth and differentiation. This type of rapid protein synthesis-independent regulation is likely to act in concert with various regulatory proteins (e.g., FLIP). Once such a regulatory protein has been produced, it will yield a more long-term and stable protection or modulation.

There are many possible targets for a direct signaling-mediated modulation of the DR signal. A protein synthesis-independent signal-based mechanism that regulates TNF-R1 sequestration has been reported. In this case, MAPK/ERK signaling was shown to phosphorylate TNF-R1 directly, thereby causing the internalization of the receptor from the surface of the cell to the cytosol and inhibition of its cytotoxic ability (51, 52). However, our study excludes the possibility of TRAIL-R internalization or down-regulation as a MAPK/ERK target, because the surface expression of the DR4 and DR5 was not altered by the activation of MAPK/ERK.

It is tempting to speculate that the protective action of MAPK/ERK would be mediated by direct or indirect phosphorylation of a DISC component. Phosphorylation of FADD has previously been indicated (53, 54). However, our results show that MAPK/ERK activation does not alter the binding of FADD or caspase-8 to the TRAIL-DISC. Therefore, it is unlikely that MAPK/ERK activation would affect the assembly of the DISC by phosphorylation of a DISC protein. Recent findings also show that FasR, DR4, and DR5 are not phosphorylated by active MAPK/ERK or by TPA (55). It seems that finding the potential MAPK/ERK target responsible for conveying the observed protection will require detailed phosphoprotein analysis far beyond the DISC.

MAPK/ERK activation inhibits the processing of caspase-8 and Bid, thereby turning off the mitochondrial amplification loop

To resolve whether the cells still maintain their normal functions, it is of great interest to know whether MAPK/ERK suppresses TRAIL-induced apoptosis at the same stage of the signaling pathway as it does in FasR-mediated apoptosis. Similarly to the FasR (34, 37), MAPK/ERK signaling seemed to suppress TRAIL-induced apoptosis before activation of caspase-8 and Bid. It has been shown that TRAIL induces caspase-8-mediated cleavage of Bid in other cell systems (56–58). Because MAPK/ERK signaling appeared to inhibit activation of both caspase-8 and Bid, we wanted to see whether inhibition of this upstream activator suppressed all traces of downstream mitochondrial activation. It has not yet been clear whether the mitochondrial pathway is involved in TRAIL-induced apoptosis. The results in this work show that Bid is rapidly cleaved with simultaneous translocation of tBid in TRAIL-stimulated cells. MAPK/ERK activation inhibits both cleavage and translocation of tBid from the cytoplasm to the mitochondria, as well as the observed loss in MMP and release of cyt *c* to the cytosol, after stimulation of the FasR and TRAIL-Rs. Normally during apoptosis, released cyt *c* binds to apoptosis protease-activating factor 1 in the cytosol to form the apoptosome where caspase-9 is activated. Caspase-9 accelerates the cleavage of caspase-8 by the proposed mitochondrial amplification loop suggested in type II cells (42). The results presented in this work show that MAPK/ERK signaling completely abrogates the engagement of mitochondria during TRAIL-induced apoptosis. Furthermore, our results indicate that the activation of TRAIL-induced cyt *c* release is truly dependent on the cleavage of Bid, because there were no traces of cyt *c* release when Bid cleavage was inhibited. Taken together, all of our results demonstrate that the inhibition of the apoptotic TRAIL signal occurs at the very proximal stages of apoptotic signaling. Inhibition at the site of death signal initiation would be a favorable way to abrogate the death signal, because the cell can thus avoid any partial damage and survive unaffected.

The elevated MAPK/ERK signaling after activation of T cells enters and inhibits the DR pathway, thereby allowing the cells to live long enough to fulfill their tasks. When the T cells are no longer needed, the MAPK/ERK activity and the levels of inhibitory proteins decrease as a consequence of insufficient activating signals, thereby allowing the cells to die by AICD. In future studies it will be of great importance to determine the molecular mechanisms and targets underlying the MAPK/ERK-mediated inhibition of apoptotic signaling in both TRAIL-induced and FasR-mediated apoptosis. Defining these targets will have great potential in treatments of various disorders related to the functions of these receptors.

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EXHIBIT K

PRODUCT DATA SHEET

ALEXIS® BIOCHEMICALS

ALX-804-297

Monoclonal Antibody to TRAIL-R1 (human) (HS101)

[anti-DR4 (human) MAb (HS101); anti-TNFRSF 10A (human) MAb (HS101); anti-CD261 (human) MAb (HS101)]

Product Numbers/Sizes

ALX-804-297A-C100	100 µg	Purified
ALX-804-297-C100	100 µg	Purified (preservative free)
ALX-804-297F-T100	100 tests	FITC

Product Specifications

CLONE:	HS101
ISOTYPE:	Mouse IgG1
IMMUNOGEN:	Recombinant human TRAIL-R1:Fc (DR4:Fc).
SPECIFICITY:	Recognizes human TRAIL-R1. Does not cross-react with human TRAIL-R2, -R3 or -R4.
APPLICATION:	Flow Cytometry Immunocytochemistry Immunoprecipitation Functional Application: Inhibition (blocks TRAIL-R1 mediated killing if applied in solution). For Western blot of whole cell lysate use PAb to TRAIL-R1 (CT) (Prod. No. PSC-1139).
PURITY:	≥95%
CONCENTRATION:	HS101 & HS101 (preservative free): 1mg/ml HS101-FITC: 0.5mg/ml
FORMULATION:	HS101: Liquid. Purified antibody in PBS containing 0.02% sodium azide. HS101 (preservative free): Liquid. Purified antibody in PBS. HS101-FITC: Liquid. 50µg of purified antibody in PBS containing 0.09% sodium azide.
SHIPPING:	SHIPPED ON BLUE ICE
LONG TERM STORAGE:	-20°C
HANDLING:	Avoid freeze/thaw cycles.

Product Specific Literature References

Caspase-10 is recruited to and activated at the native TRAIL and CD95 death-inducing signalling complexes in a FADD-dependent manner but can not functionally substitute caspase-8: M.R. Sprick, et al.; EMBO J. **21**, 4520 (2002)

Mitogen-Activated Protein Kinase/Extracellular Signal-Regulated Kinase Signaling in Activated T Cells Abrogates TRAIL-Induced Apoptosis Upstream of the Mitochondrial Amplification Loop and Caspase-8: T.S. Soderstrom, et al.; J. Immunol. **169**, 2851 (2002)

Proteasome inhibition results in TRAIL sensitization of primary keratinocytes by removing the resistance-mediating block of effector caspase maturation: M. Leverkus, et al.; Mol. Cell. Biol. **23**, 777 (2003)

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Enhanced caspase-8 recruitment to and activation at the DISC is critical for sensitisation of human hepatocellular carcinoma cells to TRAIL-induced apoptosis by chemotherapeutic drugs: T.M. Ganten, et al.; Cell Death Differ. **11 Suppl 1**, S86 (2004)

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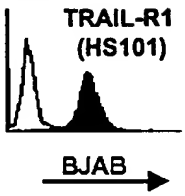

Figure: Flow cytometric detection of endogenous TRAIL-R1.

Method: BJAB cells ($1x10^6$) were stained with monoclonal antibody (MAb) HS101 against TRAIL-R1 (black histogram) or with an unspecific mouse IgG1 antibody as a control (white histogram). All primary antibodies were used at 10μg/ml, followed by biotinylated goat anti-Mouse IgG1 and streptavidin-PE.

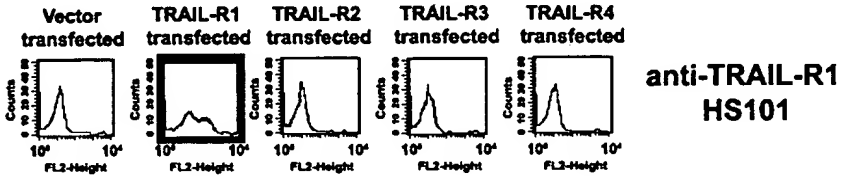

Figure: Receptor specifity of the monoclonal antibody HS101 for TRAIL-R1 (Prod. No. ALX-804-297).

Method: CV1/EBNA cells were transfected either with the empty expression vector pCDNA3 (vector control) or pCDNA3 encoding TRAIL-R1 to TRAIL-R4. Two days after transfection $1x10^6$ cells were stained with the monoclonal antibody (MAb) HS101 against TRAIL-R1 (Prod. No. ALX-804-297).

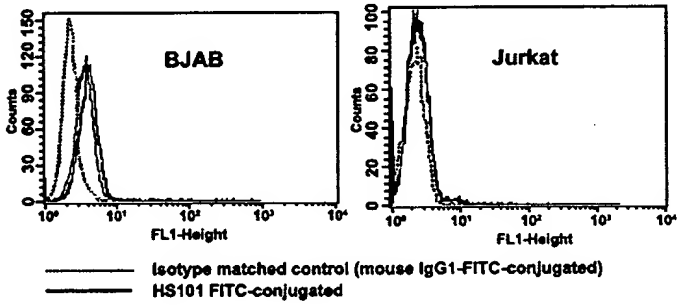

Figure: Flow cytometric detection of endogenous TRAIL-R1 surface expression on BJAB cells versus a TRAIL-R1 negative cell line, Jurkat.

Method: BJAB or Jurkat cells ($1x10^6$) were stained with FITC-conjugated monoclonal antibody (MAb) HS101 against TRAIL-R1 (Prod. No. ALX-804-297F). Antibodies were used at 5μg/ml.

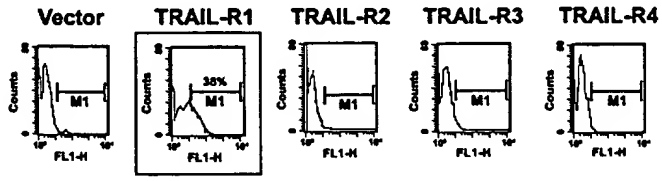

Figure: Receptor specifitiy of the FITC-conjugated MAb HS101 for TRAIL-R1 (Prod. No. ALX-804-297 F).

Method: CV1/EBNA cells were transfected either with the empty expression vector pCDNA3 (vector control) or pCDNA3 encoding TRAIL-R1 to TRAIL-R4. Two days after transfection $1x10^6$ cells were stained with FITC-conjugated MAb HS101 against TRAIL-R1. All primary antibodies were used at 5μg/ml. Transfection efficiency was about 50% in all individual transfections.

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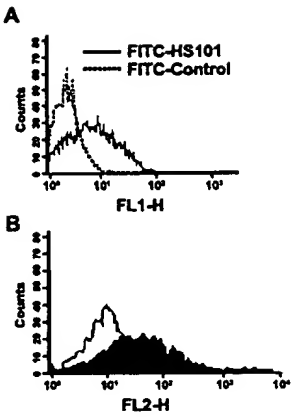

Figure A: Strong signal intensity is obtained by FITC-conjugated MAb HS101 (Prod. No. ALX-804-297 F) compared to FITC-conjugated control.

Method: CV1/EBVA cells (1 x 10^6) transiently transfected with pCDNA3 encoding TRAIL-R1, were incubated with FITC-conjugated MAb HS101 against TRAIL-R1 or FITC-conjugated matched isotype control (mouse IgG1). Antibodies were used at 5 μg/ml.
Technical Note: Signal intensity is comparable to a secondary (anti-mouse IgG1-FITC) antibody (data not shown).

Figure B: Signal intensity achieved with FITC-conjugated MAb HS101 compared to additional biotin and streptavidin-PE fluorochrome amplification step.

Method: CV1/EBVA cells were either transiently transfected with pCDNA3 encoding TRAIL-R1 (black histogram) or with empty expression vector pCDNA3 (white histogram). Cells (1 x 10^6) were first incubated with FITC-conjugated MAb HS101 (5 μg/ml), followed by biotinylated anti-mouse IgG1 and streptavidin-PE.
Technical Note: Signal increase is not significantly greater for biotin and streptavidin-PE amplification as opposed to FITC-conjugated MAb HS101 alone (Prod. No. ALX-804-297 for additional information).

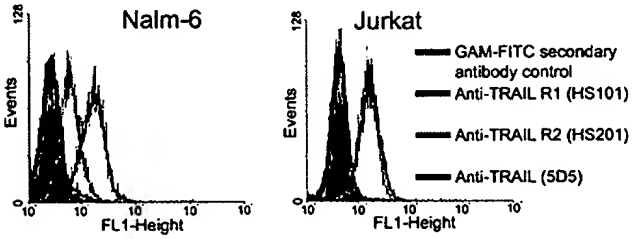

Figure: Immunodetection of endogenous TRAIL-R1 (DR4) and TRAIL-2 (DR5) and TRAIL on human hematopoietic cell lines using MAbs HS101, HS201 and 5D5 in Flow Cytometry.
Method: Antibodies were used at a final concentration of 50μg/ml in 20μl reaction per 100.000 cells; as secondary antibody FITC-conjugated goat anti-mouse IgG1 (Prod. No. ALX-211-200) at final concentration 10μg/ml in 20μl reaction was used.

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The Tumor Necrosis Factor-related Apoptosis-inducing Ligand Receptors TRAIL-R1 and TRAIL-R2 Have Distinct Cross-linking Requirements for Initiation of Apoptosis and Are Non-redundant in JNK Activation*

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Overexpression of the tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) receptors, TRAIL-R1 and TRAIL-R2, induces apoptosis and activation of NF-κB in cultured cells. In this study, we have demonstrated differential signaling capacities by both receptors using either epitope-tagged soluble TRAIL (sTRAIL) or sTRAIL that was cross-linked with a monoclonal antibody. Interestingly, sTRAIL was sufficient for induction of apoptosis only in cell lines that were killed by agonistic TRAIL-R1- and TRAIL-R2-specific IgG preparations. Moreover, in these cell lines interleukin-6 secretion and NF-κB activation were induced by cross-linked or non-cross-linked anti-TRAIL, as well as by both receptor-specific IgGs. However, cross-linking of sTRAIL was required for induction of apoptosis in cell lines that only responded to the agonistic anti-TRAIL-R2-IgG. Interestingly, activation of c-Jun N-terminal kinase (JNK) was only observed in response to either cross-linked sTRAIL or anti-TRAIL-R2-IgG even in cell lines where both receptors were capable of signaling apoptosis and NF-κB activation. Taken together, our data suggest that TRAIL-R1 responds to either cross-linked or non-cross-linked sTRAIL which signals NF-κB activation and apoptosis, whereas TRAIL-R2 signals NF-κB activation, apoptosis, and JNK activation only in response to cross-linked TRAIL.

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL),[1] also designated as APO-2 ligand, is a member of the tumor necrosis factor (TNF) family that is capable of inducing apoptosis in several cell lines (1, 2). TRAIL is widely expressed in normal cells and is highly homologous to FasL, another cytotoxic member of the TNF ligand family (1, 2). In addition to an involvement of TRAIL in natural killer cell-, dendritic cell-, and CD4+ T-cell-mediated cytotoxicity (3–6), TRAIL may also be involved in monocyte-mediated tumoricidal activity (7) and activation-induced T cell death during HIV infection (8, 9).

Currently five human TRAIL receptors belonging to the TNF receptor superfamily have been identified. Two of them, TRAIL-R1 (DR4, Ref. 10) and TRAIL-R2 (DR5, TRICK2, KILLER; see Refs. 11–18) contain a cytoplasmic death domain and transmit an apoptotic signal in response to TRAIL. Two other cellular TRAIL receptors, TRAIL-R3 (TRID, DcR1; see Refs. 11, 14, 17, 19), which is glycosylphosphatidylinositol (GPI)-linked and TRAIL-R4 (DcR2; see Refs. 20, 21), which contains a truncated death domain, bind TRAIL without activation of the apoptotic machinery and seem to antagonize the death domain-containing TRAIL receptors. In addition, osteoprotegerin, a regulator of osteoclastogenesis, is a soluble receptor for TRAIL (22). TRAIL-R1- and TRAIL-R2-mediated apoptosis occurs via activation of caspase-8 and subsequent activation of effector caspases. However, the link between the death domain-containing TRAIL receptors and activation of caspase-8 is rather undefined. Transient transfection of TRAIL-R1 leads to activation of the apoptotic machinery in Fas-associated death domain protein-deficient fibroblasts, suggesting that FADD, a death domain adapter molecule, is not required for TRAIL-R1-induced apoptosis (23, 24). On the other hand, overexpression of a dominant-negative FADD mutant was shown to block TRAIL-mediated apoptosis (12, 13, 15, 25). It has not yet been clarified whether FADD plays a specific role in TRAIL-R2-induced apoptosis or whether a closely related adapter protein is involved in TRAIL-R1- and TRAIL-R2-mediated apoptosis.

Because TRAIL is highly effective in killing cancer cell lines but has apparently no lethal effects on normal cells, TRAIL and its apoptotic receptors have attracted much attention as targets for anti-cancer therapy (26, 27). In this study, we show that TRAIL-R1 and TRAIL-R2 have different capabilities for stimulating the JNK pathway and differ also in their cross-linking requirements for activation by recombinant ligands. This is the first reported evidence of a difference between TRAIL-R1 and TRAIL-R2 signaling activities.

EXPERIMENTAL PROCEDURES

Materials—The anti-FLAG monoclonal antibody M2 was purchased from Sigma-Aldrich (Deisenhofen, Germany). Polyclonal sera specific for JNK, p65, p50, and cRel were purchased from Santa Cruz Biotechnology (Heidelberg, Germany) and protein A-Sepharose was from Amersham Pharmacia Biotech (Freiburg, Germany). The SuperFect transfection reagent was obtained from Qiagen (Hilden, Germany). TRAIL-R1-Fc and TRAIL-R2-Fc were from Alexis (Läufelfingen, Switzerland).

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[1] The abbreviations used are: TRAIL, TNF-related apoptosis-inducing ligand; JNK, c-Jun N-terminal kinase; TNF, tumor necrosis factor; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; TRAIL-R, TRAIL receptor; FACS, fluorescence-activated cell sorter; CHX, cycloheximide; EMSA, electrophoretic mobility shift assay; Z-VAD-fmk, benzyloxycarbonyl-VAD-fluoromethylketone; mAb, monoclonal antibody; IL-6, interleukin-6; GST, glutathione *S*-transferase; ELISA, enyzme-linked immunosorbent assay.

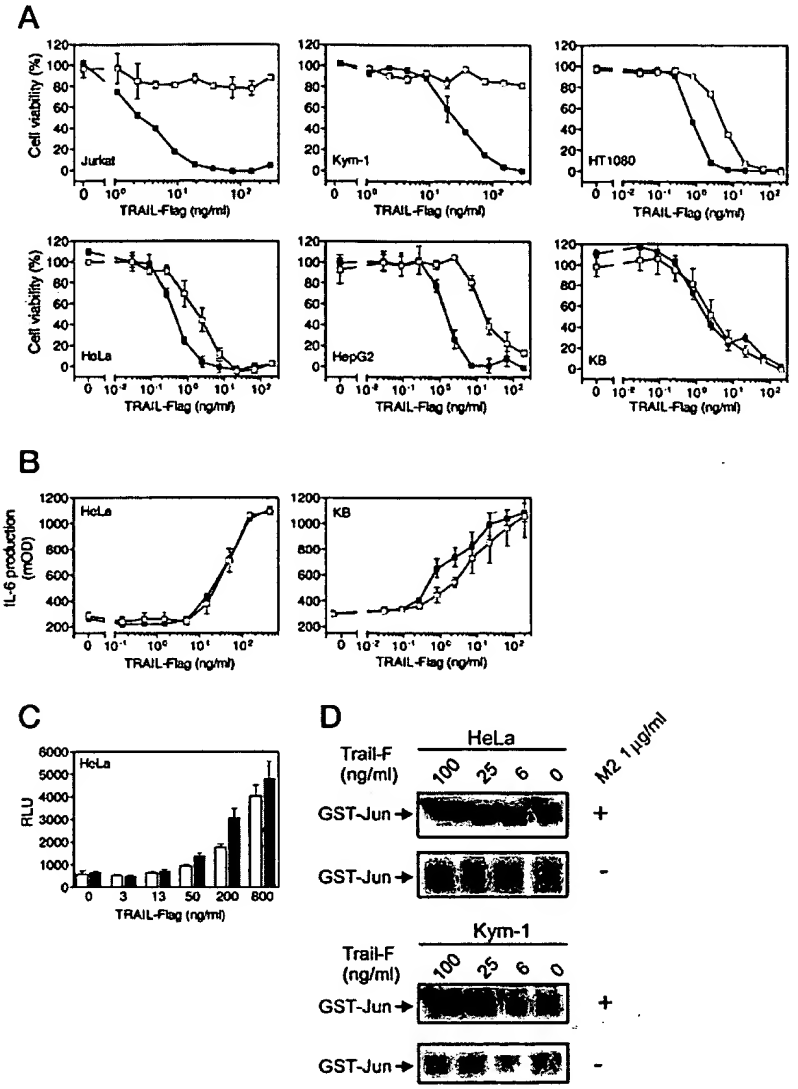

FIG. 1. **Cell type- and response-specific effects of cross-linked TRAIL-FLAG.** *A*, the indicated cell lines were analyzed for their sensitivity to FLAG-tagged sTRAIL in the presence (*filled squares*) or absence (*open squares*) of aggregating anti-FLAG antibody M2. Cell viability was determined using the MTT assay (Jurkat, Kym-1) or by staining adherent cells with crystal violet (HeLa, KB, HepG2, HT1080). The difference in absorbance between dead and living cells was in the range of 600–1000 mOD units for all cells. *B*, HeLa and KB cells were cultured overnight in 96-well plates. Cells were then incubated for 18 h with the indicated concentrations of cross-linked (*filled squares*) and non-cross-linked (*open squares*) sTRAIL-FLAG in the presence of 2.5 μg/ml CHX and 10 μM Z-VAD-fmk. Finally, IL-6 concentrations in the supernatants were determined using a commercially available ELISA kit. *C*, HeLa cells were cultured overnight in 96-well plates. The next day, cells were transfected with a 3× NF-κB-luciferase reporter plasmid and a SV40 promoter-driven β-galactosidase expression plasmid to normalize the transfection efficiency. After an additional day, cells were stimulated for 9 h with cross-linked (*filled bars*) and non-cross-linked TRAIL-FLAG (*open bars*) in the presence of CHX (2.5 μg/ml) and Z-VAD-fmk (10 μM). Finally cells were assayed for NF-κB activation. *D*, cell lysates were prepared from HeLa and Kym-1 cells that had been stimulated for 4 h with the indicated concentrations of cross-linked and non-cross-linked TRAIL-FLAG (*TRAIL-F*). JNK activity was measured by immunocomplex kinase assay with GST-c-Jun-(1–79) as substrate.

Cell Lines—HeLa, HepG2, and Jurkat cells were maintained in RPMI 1640 medium containing 5% (HeLa, HepG2) or 10% (Jurkat) heat-inactivated fetal calf serum. KB cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and HT1080 cells in Dulbecco's modified Eagle's medium-nutrient mix F12 containing 10% fetal calf serum. The Kym-1 cell line was maintained in Click RPMI 1640 medium supplemented with 10% fetal calf serum.

Generation of TRAIL-R1 and TRAIL-R2-specific IgG Preparations—Using a commercial antibody production service (Eurogentec, Seraing, Belgium), rabbits were immunized with TRAIL-R1-Fc and TRAIL-R2-Fc. For antibody purification, TRAIL-R1-Fc and TRAIL-R2-Fc were coupled to HiTrap *N*-hydroxysuccinimide (NHS)-Sepharose (Amersham Pharmacia Biotech, Freiburg, Germany) according to the manufacturer's protocol. Fc-specific antibodies were first depleted by repeated passages over human IgG1-agarose (Sigma, Deisenhofen, Germany). TRAIL-R1/R2-specific antibodies were further purified on TRAIL-R1/R2-Fc-Sepharose, eluted in 50 mM citrate/NaOH, pH 2.7, neutralized with Tris-HCl, pH 9, and dialyzed against phosphate-buffered saline. At concentrations below 500 ng/ml, we found no evidence for cross-reactivity of the anti-TRAIL receptor IgGs even upon secondary cross-linking with protein A. At higher concentrations (>1000 ng/ml) we observed a significant cross-reactivity of the anti-TRAIL-receptor IgGs that could be blocked by addition of TRAIL-R1-Fc to anti-TRAIL-R2 IgG and vice versa (data not shown).

Cytotoxic Assays—50,000 (Jurkat), 30,000 (HepG2, KB), 20,000 (HeLa, HT1080) or 15,000 Kym-1 cells were grown overnight in 100 μl of culture medium in 96-well plates. The cells were then treated for 16 h with FLAG-tagged TRAIL, TRAIL-M2-complex, anti-TRAIL-R1- and anti-TRAIL-R2-IgG. Cell death assays with HepG2, HeLa, KB, and HT1080 cells were performed in the presence of 2.5 μg/ml cycloheximide (CHX). TRAIL-M2-complex was generated by mixing the respective concentration of FLAG-tagged TRAIL with the anti-FLAG monoclonal antibody M2 to a final concentration of 1 μg/ml of antibody. After a 15-min incubation at room temperature, the TRAIL-M2-complex was transferred to the cells. TRAIL-R1 and TRAIL-R2 IgG were added in the presence of 1 μg/ml protein A (Sigma, Deisenhofen, Germany). Cell viability was determined using the MTT method (Jurkat, Kym-1) or crystal violet staining (HepG2, HeLa, KB, HT1080) as described previously (28, 29).

Immunocomplex JNK Assay—N-terminal c-Jun kinase assays were performed upon immunoprecipitation of JNK1 using a rabbit antiserum (Santa Cruz Biotechnology, Heidelberg, Germany). GST-c-Jun was used as substrate in an *in vitro* kinase assay as described previously (30).

Transient Reporter Gene Assays—For transient reporter gene assays, 20,000 HeLa cells were seeded in 96-well tissue culture plates, and the following day the cells were transfected with a 3× NF-κB-luciferase reporter plasmid (15% transfected DNA), a SV40 promoter-driven β-galactosidase expression plasmid (5% transfected DNA) to normalize the transfection efficiency, and empty vector (80% transfected DNA). Transfections were performed with SuperFect reagent according to the manufacturer's recommendations (Qiagen, Hilden, Germany). After a 1-day recovery, cells were treated with TRAIL, TRAIL-M2-complex, CHX, and Z-VAD-fmk as indicated, harvested in phosphate-buffered saline, and then luciferase and β-galactosidase activities were determined using the Galacto-Light Plus reporter gene assay kit (Perkin Elmer, Nieuwerkerk, The Netherlands) and a Lucy2 96-well luminometer (Anthos, Krefeld, Germany).

Determination of Interleukin-6 Production—Cells (1.5×10^4 per well) were seeded in triplicates in 96-well tissue culture plates in 100 μl of Click RPMI 1640 and cultured overnight. The following day the cells were treated with the reagents of interest as indicated for an additional 12–24 h. Then the supernatants were removed, cleared by centrifuga-

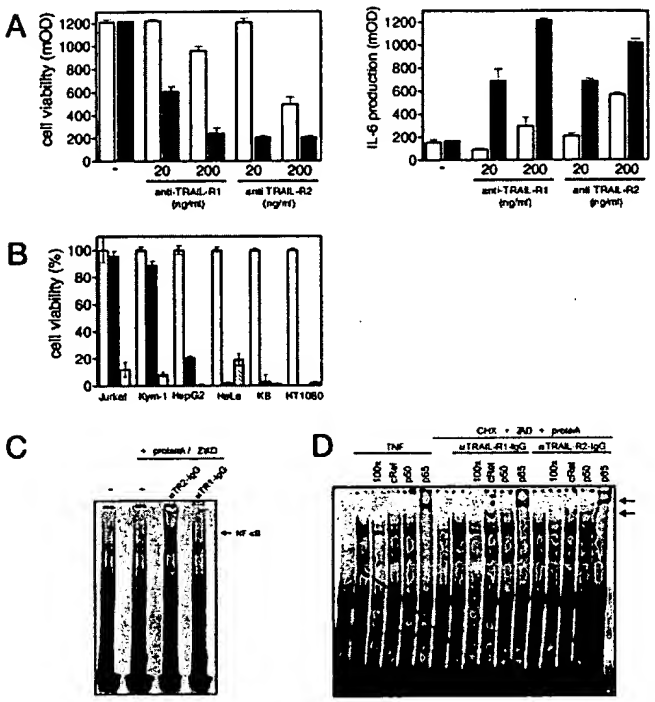

FIG. 2. *A*, protein A cross-linking enhanced the agonistic capacity of anti-TRAIL-R1 and -R2 IgG. HepG2 cells were incubated overnight with the indicated concentration of anti-TRAIL-R1 IgG and anti-TRAIL-R2 IgG, respectively, with (*filled bars*) or without (*open bars*) previous aggregation with protein A in the presence of 2.5 μg/ml CHX. Cell viability was measured by crystal violet staining. In addition, HeLa cells were treated in the same way with the anti-TRAIL-receptor IgGs in the presence of 2.5 μg/ml CHX and 10 μM Z-VAD-fmk, and supernatants were analyzed for production of IL-6. *B*, cytotoxic effects of anti-TRAIL-R1 IgG and anti-TRAIL-R2 IgG. Various cell lines were incubated overnight with protein A cross-linked anti-TRAIL-R1 IgG (*filled bars*) or anti-TRAIL-R2 IgG (*hatched bars*) or were left untreated (*open bars*). The next day, cell viability was determined by the MTT assay (Jurkat, Kym-1) or by staining with crystal violet (HeLa, KB, HepG2, HT1080). To allow induction of apoptosis, HepG2, HeLa, KB, or HT1080 cells were treated in the presence of 2.5 μg/ml CHX. *C*, Kym-1 cells were treated with anti-TRAIL-R1 or anti-TRAIL-R2 IgG (*αTR1-IgG* or *αTR2-IgG*, respectively; 200 ng/ml) and protein A (1 μg/ml) with Z-VAD-fmk (*ZVAD*, 20 μM). After 3 h, cells were harvested and analyzed for NF-κB activation by EMSA. *D*, HeLa cells were treated as indicated with, anti-TRAIL-R1 and anti-TRAIL-R2 IgG (200 ng/ml), protein A (1 μg/ml), CHX (2.5 μg/ml), and Z-VAD-fmk (20 μM) for 3 h. As a control, cells were also treated with 10 ng/ml TNF for 30 min. Supershift analyses were performed as described under "Experimental Procedures." Supershifted complexes of the NF-κB oligonucleotide and p65, p50, or cRel, respectively, are indicated by *arrows*.

tion (15,000 rpm, 10 min, 4 °C) and interleukin-6 concentrations were determined using a commercially available ELISA kit (PharMingen, Hamburg, Germany).

FACS Staining—Cells were stained for TRAIL-R1, TRAIL-R2, TRAIL-R3, and TRAIL-R4 expression in 100 μl of FACS buffer (phosphate-buffered saline, 5% fetal calf serum, 0.1% NaN_3) with 5 μg/ml anti-TRAIL-R1 mAb M271 (IgG2a), anti-TRAIL-R2 mAb M413 (IgG1), anti-TRAIL-R3 mAb M430 (IgG1) and anti-TRAIL-R4 mAb M445 (IgG1), respectively, or the respective control IgG, followed by fluorescein isothiocyanate-labeled anti-mouse antibody (5 μg/ml). FACS analyses were performed with a FACStar plus instrument (Becton Dickinson, San Jose, CA).

EMSA Analysis of NF-κB Activation—HeLa and Kym-1 cells (10^6) were seeded in 60-mm cell culture dishes and cultivated overnight to allow adherence. The next day the cells were stimulated for 3 h with the indicated combinations of anti-TRAIL-R1 and anti-TRAIL-R2 IgG, protein A (1 μg/ml), Z-VAD-fmk (20 μM) and CHX (2.5 μg/ml). Nuclear extracts were prepared as described previously (31), and EMSA analyses were performed using a standard procedure with a high pressure liquid chromatography-purified NF-κB-specific oligonucleotide (5'-ATC AGG GAC TTT CCG CTG GGG ACT TTC CG-3'), end-labeled with [^{32}P]ATP. Finally, samples were separated by native polyacrylamide gel

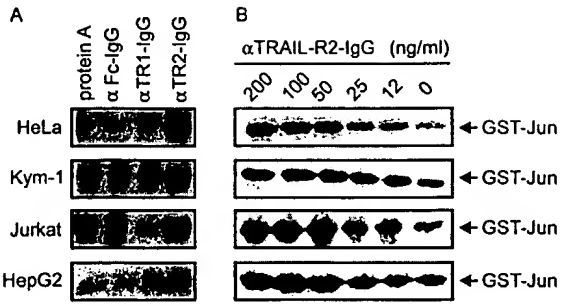

FIG. 3. **TRAIL-R2 but not TRAIL-R1 signals JNK activation.** *A*, cell lysates were prepared from Kym-1, Jurkat, HeLa, and HepG2 cells that had been stimulated for 4 h with protein A cross-linked (1 μg/μl) anti-TRAIL-R1 IgG and anti-TRAIL-R2 IgG, each at 200 ng/ml in the absence of CHX. For control purposes lysates from cells treated with protein A and non-relevant IgG were also analyzed. JNK activity was measured by immunocomplex kinase assay with GST-c-Jun-(1–79) as a substrate. *B*, dose dependence of anti-TRAIL-R2 IgG-induced JNK activation. Kym-1, Jurkat, HeLa, and HepG2 cells were stimulated with the indicated concentrations of protein A cross-linked anti-TRAIL-R2 IgG for 4 h, and lysates were analyzed for JNK activity by immunocomplex kinase assays.

electrophoresis in low ionic strength buffer. For supershift analyses, 10 μl of the nuclear extracts (1 μg/μl protein) were incubated on ice for 1 h with 1 μg of polyclonal antibodies specific for p65, p50, and cRel (Santa Cruz Biotechnology, Heidelberg, Germany). Then the formed complexes were incubated with 2 μl of 5× binding buffer (500 mM KCl, 50 mM Tris-HCl, pH 7.4, 25 mM $MgCl_2$, 50% glycerol, 5 mM dithiothreitol) and 2 μl of poly(dI-dC) (2 mg/ml) in a final volume of 20 μl. NF-κB DNA-binding activity was again analyzed by native polyacrylamide gel electrophoresis and phosphorimaging (Storm 860; Amersham Pharmacia Biotech, Freiburg, Germany).

RESULTS AND DISCUSSION

Most ligands of the TNF family are either membrane-bound or proteolytically processed into soluble proteins. Evidence suggests that artificial cross-linking of soluble ligands mimics the distinct biological activities of the corresponding membrane-bound ligands. For example, we have recently shown that the cytotoxic activity of FLAG-tagged human Fas ligand (sFasL), was increased by >1000-fold in response to cross-linking with the anti-FLAG monoclonal antibody M2. Notably, this increased activity was comparable with the cytotoxic potency of membrane-bound FasL (32). Further, activation of TNF-R2-dependent signaling pathways by soluble FLAG-tagged TNF was strongly increased by multimerization of this ligand by the anti-FLAG monoclonal antibody M2. In accordance with that, we have previously shown that membrane-bound, but not soluble TNF, is the prime activating ligand for TNF-R2 (33, 34), suggesting that cross-linked and membrane-bound ligands have analogous effects on this receptor. Using various cell lines we have therefore tested whether a recombinant soluble FLAG-tagged form of TRAIL (sTRAIL) required cross-linking for its activity.

We found that several cell lines, *e.g.* Jurkat and Kym-1, designated in the following as group I cells, were killed by physiological amounts (<200 ng/ml) of sTRAIL only in the presence of secondary cross-linking by the anti-FLAG monoclonal antibody M2 (Fig. 1*A*). However, we also identified a second set of cell lines designated in the following as group II cells that were efficiently killed by non-cross-linked sTRAIL (HeLa, HepG2, HT1080, and KB; Fig. 1*A*). The group II cell lines Hela and KB were also tested with respect to the cross-linking requirements of sTRAIL for non-apoptotic signaling. As shown in Fig. 1, cross-linked and non-cross-linked sTRAIL both have a comparable capacity to induce IL-6 production (Fig. 1*B*) and elicited comparable NF-κB activation in a reporter gene assay (Fig. 1*C*). The magnitude of NF-κB activation and IL-6

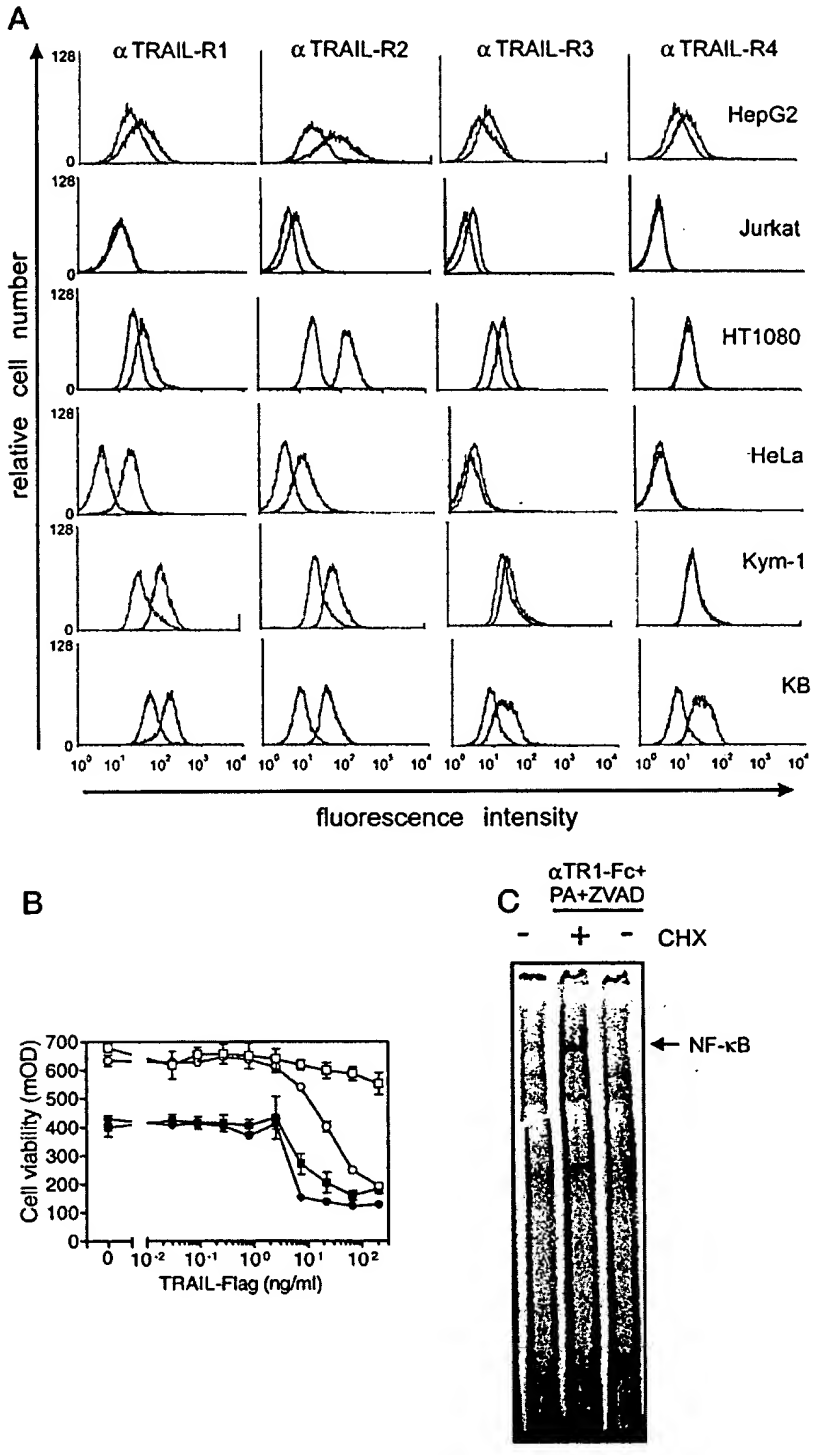

FIG. 4. *A*, analysis of TRAIL receptor expression on various cell lines by FACS. *B*, effect of CHX on the killing of Kym-1 cells by cross-linked and non-cross-linked sTRAIL. Kym-1 cells were cultured overnight in 96-well plates. Cells were then incubated for additional 18 h with the indicated concentrations of cross-linked (*circles*) and non-cross-linked (*squares*) sTRAIL in the presence (*filled symbol*) or absence (*open symbol*) of 2.5 μg/ml CHX. Cell viability was measured by the MTT assay. *C*, Kym-1 cells were treated with anti-TRAIL-R1 IgG (200 ng/ml), protein A (1 μg/ml), and Z-VAD-fmk (*ZVAD*, 20 μM) in the presence or absence of 2.5 μg/ml CHX. After 3 h, cells were harvested and analyzed for NF-κB activation with EMSA.

production induced by cross-linked and non-cross-linked sTRAIL was similar to that obtained by TNF stimulation (data not shown). In all group II cells investigated, TRAIL-induced apoptosis and activation of NF-κB were dependent on the presence of CHX. Activation of the NF-κB pathway is inhibited by caspase-dependent mechanisms during apoptosis (35–38). Thus, TRAIL-induced activation of NF-κB was therefore only observed in group II cells when in addition to CHX a caspase inhibitor (Z-VAD-fmk) was present (data not shown). However, in group I cells, NF-κB activation was found in the absence of CHX, provided that apoptosis was again inhibited by Z-VAD-fmk (data not shown). Notably, when we analyzed TRAIL-mediated JNK activation in group I (Kym-1) and II cells (HeLa), we found in both cell lines a requirement for cross-linked sTRAIL (Fig. 1*D*). As already outlined above, in group II cell lines, sTRAIL activated NF-κB only in the presence of CHX/Z-VAD-fmk and induced cell death only if CHX was present. However, activation of the JNK pathway by cross-linked TRAIL occurred in the absence of CHX and was therefore not linked to cell death.

To analyze whether the requirement for cross-linked sTRAIL correlated with a differential utilization of TRAIL-R1 and TRAIL-R2, we reexamined the cells described above using purified IgG fractions of agonistic TRAIL-R1- and TRAIL-R2-specific antisera. The agonistic activity of anti-TRAIL-R1 IgG and anti-TRAIL-R2 IgG, respectively, was significantly in-

creased upon aggregation with protein A, with respect to death induction and up-regulation of IL-6 production (Fig. 2*A*). At the concentrations used in this study (<200 ng/ml) the IgG fractions were not cross-reactive. Using the agonist anti-TRAIL-receptor IgGs, we found that group I cells were exclusively killed by anti-TRAIL-R2 IgG, whereas group II cells were sensitive for stimulation with both anti-TRAIL-R1 and anti-TRAIL-R2 IgG (Fig. 2*B*). Moreover, in the group II cell line HeLa, both IgG preparations induced NF-κB activation whereas in the group I cell line Kym-1 only anti-TRAIL-R2 IgG but not anti-TRAIL-R1 IgG was able to activate NF-κB (Fig. 2, *C* and *D*). Again in HeLa cells treatment with CHX and Z-VAD-fmk was necessary to elicit the NF-κB response, whereas in Kym-1 cells NF-κB activation only required inhibition of the apoptotic pathway. Supershift analyses in HeLa cells revealed that TRAIL-R1 and TRAIL-R2 engaged the NF-κB family members p65, p50, and cRel in a comparable manner to TNF-R1 (Fig. 2*D*). In group I as well as in group II cell lines the first signs of NF-κB DNA-binding activity were detectable 1 to 2 h upon TRAIL receptor stimulation whereas TNF induced NF-κB DNA-binding activity within 15–30 min. NF-κB DNA-binding activity induced by both cytokines sustained for several hours in both type of cells (data not shown). Importantly, the JNK pathway was triggered in group I and II cell lines by stimulation of TRAIL-R2 but not by stimulation of TRAIL-R1 (Fig. 3). To our knowledge, this is the first reported difference in the signaling capacities of the two death domain-containing TRAIL receptors.

Based on these results, it is evident that group II cells must co-express both death domain-containing TRAIL receptors, whereas group I cells either express no TRAIL-R1 or this molecule was silenced in some way under the conditions used in our study. As shown in Fig. 4*A*, all cells investigated with the exception of Jurkat cells were positive for TRAIL-R1 and TRAIL-R2 expression in FACS analysis. In addition, with the exception of Jurkat and HeLa cells, all investigated cell lines express at least one of the TRAIL decoy receptors (TRAIL-R3, TRAIL-R4). Nevertheless, in all cases the expression of the decoy receptors was rather low compared with TRAIL-R1 and TRAIL-R2 expression, which is consistent with the TRAIL-sensitivity of these cell lines. In light of the expression data it became clear that the group I cell line Jurkat did not respond to anti-TRAIL-R1 IgG (or non-cross-linked sTRAIL) as TRAIL-R1 is not significantly expressed on this cell line. However, in the case of the TRAIL-R1-expressing Kym-1 cell line, it is obvious that TRAIL-R1 signaling has to be negatively regulated (by an unknown mechanism). As in group II cells, because low concentrations of the metabolic inhibitor CHX were necessary to allow TRAIL-R1-mediated NF-κB activation and induction of cell death, we tested the signaling capacity of TRAIL-R1 in this group I cell line also in the presence of CHX. In fact, Kym-1 cells became sensitive to anti-TRAIL-R1 IgG (data not shown) and non-cross-linked sTRAIL (Fig. 4*B*) in the presence of CHX. Moreover, whereas in the absence of CHX only stimulation of TRAIL-R2 activated the NF-κB pathway (see Fig. 2*C*), stimulation of TRAIL-R1 also induced NF-κB activation provided that CHX and Z-VAD-fmk were added (see Fig. 4*C*). Because of the high cytotoxicity of CHX, putative effects of this compound on TRAIL receptor-induced apoptosis, cells could not be examined in Jurkat cells (data not shown). Our observations may suggest the existence of two CHX-sensitive factors or pathways. The first one is active in group II cells to prevent TNF-, FasL-, and TRAIL-mediated cell death. The second ligand may specifically block TRAIL-R1 pathways in group I cells. It is noteworthy that TRAIL-R2-mediated JNK activation occurred in group I and group II cells in the absence of CHX, clearly demonstrating the ability of TRAIL-R2 to transmit specific signals in the absence of cell death. It is possible that TRAIL-R2 is also important for non-apoptotic signal transduction. This may involve the activation of c-Jun and other JNK- or NF-κB related downstream responses, which regulate proliferation and differentiation in normal cells. The apoptotic function of TRAIL-R2, which is cryptic in normal cells, may only be dominantly revealed in transformed cells. Although JNK was only activated *via* endogenous TRAIL-R2 but not endogenous TRAIL-R1, we also noted that transient overexpression of both TRAIL-R1 and TRAIL-R2 activated JNK in a ligand-independent fashion (data not shown). We can therefore not completely exclude the possibility that endogenous TRAIL-R1 might activate JNK in some circumstances.

In conclusion, our data suggest that TRAIL-R1 responds to cross-linked and non-cross-linked TRAIL to signal NF-κB activation and apoptosis, whereas TRAIL-R2 signals NF-κB activation, apoptosis, and JNK activation in response to cross-linked TRAIL only. We hypothesize that the requirement of cross-linked sTRAIL reflects the requirement of TRAIL-R2 for membrane-bound TRAIL.

Acknowledgment—The authors thank David Lynch, Immunex Corporation, Seattle, WA for supplying us with TRAIL receptor-specific mAbs M271, M413, M430, and M445.

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EXHIBIT M

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Product:	Anti-TRAIL Receptor 3, N-Terminal (73-103), Human (Goat)	**Cat. No.:**	616383
		Lot No.:	B27674
		Size:	100 µl
Synonyms:	Anti-TRID; Anti-Trail-R3	**Storage:**	-70°C
		Usage:	Working dilution: 1:300 for immunocytochemistry; 1:1000 for immunoblotting (ECL); ELISA

Product Specifications:

Source: Goat

General Description: IgG fraction purified by peptide affinity column chromatography. Recognizes the human TRAIL Receptor-3.

Immunogen: Immunogen was a synthetic peptide corresponding to amino acid residues 73-103 (EVPQQTVAPQQQRHSFKGEECPAGSHRSEHTC) of the human TRAIL Receptor 3 protein. This region of the protein has low sequence homology to TRAIL Receptor 1.

Buffer: 10 mM Potassium phosphate, 140 mM NaCl, pH 7.2, with 1 mg/ml BSA as a stabilizer.

Preservatives: 0.1% sodium azide

Specificity: Recognizes a doublet of 32-35 kDa corresponding to the human TRAIL Receptor-3 antibody in Jurkat (T-cell), and 293 (embryonic kidney) human cell lines. Immunoblots using lysates of the human myelocytic cell line HL60 were negative. Binds to a 32-33 kDa band in mouse splenocytes that may represent the putative mouse TRAIL Receptor-3 protein. Exhibits <2% cross reactivity by ELISA to the TRAIL Receptor-2 peptide for the same region, and does not recognize the 50-53 kDa band corresponding to TRAIL Receptor-2 by western blotting.

Special Instructions: Avoid freeze/thaw cycles.

Potency/Assay Conditions: Variables associated with assay conditions will dictate the proper working dilution.

References:

Degli-Esposti, M.A., et al. 1997. *J. Exp. Med.* **186,** 1165.

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Revised: 9-Nov-99

EXHIBIT N

Anti-DcR2 Polyclonal Antibody #B50005

Full activity guaranteed through February 2009.
This certificate is a declaration of analysis at the time of manufacture.

*B50005____024056
7/*

Lot Number 0240567

Certified By Bryan Macilko

Quality Controlled By Jackie Jaskula

Quantity 100 µg

Description

Apoptosis is induced by certain cytokines including TNF and Fas ligand in the TNF family through their death domain containing receptors. TRAIL/Apo2L is a new member of the TNF family and induces apoptosis of a variety of tumor cell lines. DR4 and DR5 are the recently identified functional receptors for TRAIL, and DcR1/TRID is a decoy receptor. Another member of the TRAIL receptor family was more recently identified and designated DcR2, TRAIL/R4, or TRUNDD. DcR2 has an extracellular TRAIL-binding domain but lacks intracellular death domain and does not induce apoptosis. Like DR4 and DR5, DcR2 transcript is widely expressed in normal human tissues. Overexpression of DcR2 attenuated TRAIL-induced apoptosis.

Antigen

Rabbit anti-DcR2 polyclonal antibody was raised against a peptide corresponding to amino acids 249 to 263 of human DcR2 precursor.

Western blot	1:1000–2000
Isotype	Rabbit Ig
Specificity	human
Molecular Weight	35 kDa

Anti-DcR2 Polyclonal Antibody #B50005

Full activity guaranteed through February 2009.
This certificate is a declaration of analysis at the time of manufacture.

*B50005____024056
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Lot Number 0240567

Certified By Bryan Macilko

Quality Controlled By Jackie Jaskula

Quantity 100 µg

Description

Apoptosis is induced by certain cytokines including TNF and Fas ligand in the TNF family through their death domain containing receptors. TRAIL/Apo2L is a new member of the TNF family and induces apoptosis of a variety of tumor cell lines. DR4 and DR5 are the recently identified functional receptors for TRAIL, and DcR1/TRID is a decoy receptor. Another member of the TRAIL receptor family was more recently identified and designated DcR2, TRAIL/R4, or TRUNDD. DcR2 has an extracellular TRAIL-binding domain but lacks intracellular death domain and does not induce apoptosis. Like DR4 and DR5, DcR2 transcript is widely expressed in normal human tissues. Overexpression of DcR2 attenuated TRAIL-induced apoptosis.

Antigen

Rabbit anti-DcR2 polyclonal antibody was raised against a peptide corresponding to amino acids 249 to 263 of human DcR2 precursor.

Western blot	1:1000–2000
Isotype	Rabbit Ig
Specificity	human
Molecular Weight	35 kDa

Anti-DcR2 Polyclonal Antibody #B50005

Full activity guaranteed through February 2009.
This certificate is a declaration of analysis at the time of manufacture.

*B50005____024056
7/*

Lot Number 0240567

Certified By Bryan Macilko

Quality Controlled By Jackie Jaskula

Quantity 100 µg

Description

Apoptosis is induced by certain cytokines including TNF and Fas ligand in the TNF family through their death domain containing receptors. TRAIL/Apo2L is a new member of the TNF family and induces apoptosis of a variety of tumor cell lines. DR4 and DR5 are the recently identified functional receptors for TRAIL, and DcR1/TRID is a decoy receptor. Another member of the TRAIL receptor family was more recently identified and designated DcR2, TRAIL/R4, or TRUNDD. DcR2 has an extracellular TRAIL-binding domain but lacks intracellular death domain and does not induce apoptosis. Like DR4 and DR5, DcR2 transcript is widely expressed in normal human tissues. Overexpression of DcR2 attenuated TRAIL-induced apoptosis.

Antigen

Rabbit anti-DcR2 polyclonal antibody was raised against a peptide corresponding to amino acids 249 to 263 of human DcR2 precursor.

Western blot	1:1000–2000
Isotype	Rabbit Ig
Specificity	human
Molecular Weight	35 kDa

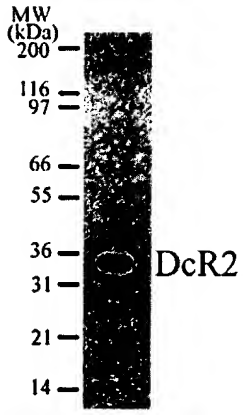

Figure 1. Western blot detection of DcR2. Western blot analysis of 50 µg of whole HeLa cell lysate with anti-DcR2 at a 1:1000 dilution.

Storage Buffer

The antibody is supplied in PBS with 0.02% sodium azide.

Shipping Conditions

The Anti-DcR2 antibody is shipped with an ice pack.

Storage Conditions

The antibody should be stored at –20°C for long term storage and can be stored at 4°C for up to six months. Avoid repeated freeze-thaws.

Testing Conditions

DcR2-expressing HeLa cell lysates are resolved by PAGE and analyzed by Western blotting using the recommended antibody dilution.

Limited Product Warranty

This warranty limits our liability to replacement of this product. No other warranties of any kind, express or implied, including, without limitation, implied warranties of merchantability or fitness for a particular purpose, are provided by Stratagene. Stratagene shall have no liability for any direct, indirect, consequential, or incidental damages arising out of the use, the results of use, or the inability to use this product.

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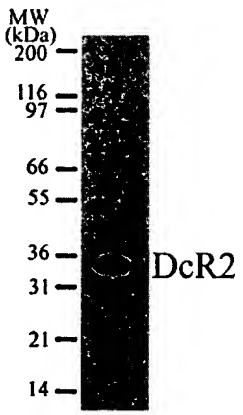

Figure 1. Western blot detection of DcR2. Western blot analysis of 50 µg of whole HeLa cell lysate with anti-DcR2 at a 1:1000 dilution.

Storage Buffer

The antibody is supplied in PBS with 0.02% sodium azide.

Shipping Conditions

The Anti-DcR2 antibody is shipped with an ice pack.

Storage Conditions

The antibody should be stored at –20°C for long term storage and can be stored at 4°C for up to six months. Avoid repeated freeze-thaws.

Testing Conditions

DcR2-expressing HeLa cell lysates are resolved by PAGE and analyzed by Western blotting using the recommended antibody dilution.

Limited Product Warranty

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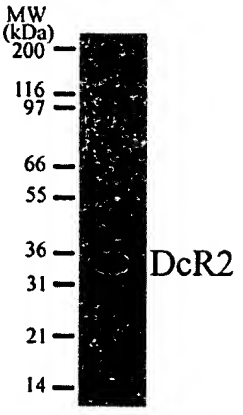

Figure 1. Western blot detection of DcR2. Western blot analysis of 50 µg of whole HeLa cell lysate with anti-DcR2 at a 1:1000 dilution.

Storage Buffer

The antibody is supplied in PBS with 0.02% sodium azide.

Shipping Conditions

The Anti-DcR2 antibody is shipped with an ice pack.

Storage Conditions

The antibody should be stored at –20°C for long term storage and can be stored at 4°C for up to six months. Avoid repeated freeze-thaws.

Testing Conditions

DcR2-expressing HeLa cell lysates are resolved by PAGE and analyzed by Western blotting using the recommended antibody dilution.

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